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DEVELOPMENTAL STUDIES

Conducted By Diagnostic Services National Animal Disease Laboratory Fiscal Year 1967

(And Abstracts of Related Published Articles)

Agricultural Research Service
U.S. DEPARTMENT OF AGRICULTURE

PREFACE

The development and the improvement of existing diagnostic procedures are an integral part of the operations of a competent diagnostic service laboratory. The abstracts of published reports and of unpublished project reports contained herein provide a reference source of the achievements made by Diagnostic Services, Animal Health Division, Agricultural Research Service, U.S. Department of Agriculture, at Ames, Iowa, during fiscal year 1967 in this area of laboratory activity.

These reports will be of value to veterinary diagnosticians, epidemiologists, and regulatory officials in achieving the common goal of providing increased diagnostic capabilities to the livestock and poultry industries.

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CONTENTS

CONTENTS—continued

Developmental Studies Conducted by Diagnostic Services, National Animal Disease Laboratory, Fiscal Year 1967, and Abstracts of Related Published Articles

BRUCELLOSIS

Project Reports

Brown, G. M. Evaluation of *Brucella abortus* Strain 45/20 Bacterins Prepared with Various Adjuvants.

Abstract

The potentiating effects of five different adjuvants upon the immunogenic properties of a heat-inactivated nonagglutinogenic strain (45/20) of *Brucella abortus* was determined for guinea pigs. The degree of protection afforded immunized animals was directly related to the amount of tissue response produced by the adjuvant. When this response was relatively severe, the protection was comparable to that conferred by living *Br. abortus* Strain 19 against challenge by a virulent strain (2308) of *Br. abortus.* The degree of potentiation by a adjuvants producing little or no gross tissue response was negligible.

Introduction

The widespread use of *Brucella abortus* Strain 19 as a prophylactic measure has contributed a great deal to the brucellosis eradication program in the United States. It has, however, certain limitations that are well recognized. Most important of these has been the confusion created in the interpretation of serological results of vaccinated animals subjected to serological tests.

British workers attempted to circumvent this problem by employing a relatively avirulent nonagglutinogenic strain (45/20) of *Br. abortus* as a live immunizing agent (7, 8, 15, 19). Because strain 45/20 reverted to a fully virulent strain on passage through cattle (19), later efforts were directed toward the development of a bacterin from this strain. McDiarmid and Sutherland (14) reported that a bacterin prepared with strain 45/20 appeared to surpass the live vaccine in its ability to protect guinea pigs against challenge by a virulent strain 544.

McDiarmid (13) demonstrated that a bacterin prepared with strain 45/20 was comparable to strain 19 in providing protection to cattle against challenge by virulent strain 544 providing challenge was not too severe. Objectionable features of this bacterin were the size of the dose and the degree of tissue reaction produced by the adjuvant.

This study was designed to evaluate the potentiating effects of different adjuvants on the immunogenic properties of heat inactivated *Br. abortus* strain 45/20. The main criteria for this evaluation was the relative ability of these bacterins, as compared to *Br. abortus* strain 19 vaccine, to provide protection in guinea pigs challenged with virulent *Br. abortus* strain 2308.

The parameters considered in this evaluation were: (1) Seroagglutination titers, (2) spleen/body weight ratios, (3) colony counts from cultured spleens, (4) gain or loss of body weight, and (5) gross lesions produced.

Materials and Methods

Immunizing Products.—These included bacterins A, B, and C prepared with adjuvants from commercial sources;[1] bacterin D, a water in oil emulsion prepared in this laboratory; bacterin E, a commercial product produced in The Netherlands;[2] and the U.S. Department of Agriculture strain 19 liquid vaccine.

Animals.—The 360 male guinea pigs, each weighing 400 to 500 grams, were divided into two groups of 180 guinea pigs—a 2-month group and a 7-month group. Each major group was further subdivided into 12 groups of 15 guinea pigs each. The corresponding subgroups of the two major groups received identical treatments except that the 2-month group was challenged 2 months following completion of their immunization schedule and the 7-month group was challenged 7 months following completion of their immunization schedule. Appropriate control groups were included in each of the major groups.

[1] Adjuvants A and B—Fort Dodge Laboratories, Fort Dodge, Iowa. Adjuvant C (Algivant)—Consolidated Laboratories, Inc., Chicago Heights, Ill.

[2] Research Laboratories of N. V. Philips—Duphar, Weesp, Netherlands.

All guinea pigs were housed in plastic cages with six animals per cage. They were fed commercial guinea pig pellets supplemented with fresh lettuce twice weekly throughout the study.

Organisms.—*Brucella abortus* strain 45/20 was grown and maintained on tryptose throughout the study. The colonial index (10) was judged to be 50 percent SI_3 and 50 percent I-IR. *Br. abortus* strain 19 was judged to have a colonial index of 100 percent SI_2.

Br. abortus strain 2308 was the challenge strain. It had been passed in guinea pigs and transferred three times on potato-infusion agar before use for challenge. The colonial index was judged to be 100 percent SI_2.

Preparation of Immunizing and Challenging Agents.—Strain 45/20 was washed from the surface of the media contained in Roux flasks with phosphate buffered saline, pH 6.4, and standardized to an optical density (O.D.) of 18 percent T at 620 mμ on a Bausch and Lomb Spectronic 20.[3] This suspension had a viable count of 4.35×10^9 cells/per ml. and the suspension used in preparing bacterins A, B, C, and D.

The standardized suspension was inactivated by heating in a water bath at 65° C. for 1 hour.

Bacterins A and B were prepared in an identical manner. One hundred ml. of the cell suspension was added to the adjuvant and the bottle placed on a mechanical shaker for 30 minutes.

Bacterin C was prepared by mixing equal volumes of the adjuvant and cell suspension in such a manner as to give a homogeneous suspension.

Bacterin D was prepared by adding 10 ml. of Arlacel A[4] to 90 ml. of Bayol 55.[5] This was mixed for a few seconds at a low speed in a blender. An equal volume of cell suspension was added in increments of 25 ml. each. Brief periods of blending followed the addition of each increment. After the entire volume of cell suspension had been added, blending was continued until the emulsion gave a satisfactory drop test in water.

Bacterin E was a commercially prepared product reported to have 150×10^9 cells/ml.

Strain 19 liquid vaccine was standardized to an O.D. of 47 percent T at 620 mμ on a Spectronic 20. This suspension had a viable count of 2.02×10^9 cells/ml.

A 48-hour culture of strain 2308 was washed from the surface of a potato-infusion-agar slant with phosphate-buffered saline and standardized to an O.D. of approximately 72 percent T at 620 mμ on a Spectronic 20. This was estimated to give a suspension of 1×10^9 cells/ml. Three hundredfold dilutions of this suspension were made in 1-percent peptone broth. One ml. of the final dilution was used to challenge each guinea pig. The 2-month inoculum had a viable count of 1.09×10^3 and the 7-month inoculum a viable count of 5×10^2.

Immunization Schedules.—Subcutaneous and intramuscular routes were employed with bacterins A, B, C, and D. Two 0.5 ml. injections were given at 2-week intervals. Subcutaneous injections were alternated between the right and left axillary region and intramuscular injections were alternated between the right and left thigh.

Bacterin E was recommended for intramuscular injection only in the bovine; hence, no other routes were employed with this bacterin. As with the other bacterins, two 0.5 ml. injections were given at 2-week intervals.

Strain 19 was given as a single 0.5 ml. injection subcutaneously in the inguinal region to coincide with the first injection of bacterin.

Necropsy Schedules and Spleen Counts.—The necropsy schedules of the two major groups were identical with one-third of the guinea pigs of each of the subgroups being necropsied on postchallenge day 34, one-third on postchallenge day 35, and the reamining one-third on postchallenge day 36.

Guinea pigs were given 0.5 ml. of Nembutal[6] intrathoracically, bled by cardiac puncture, necropsied, and examined for gross lesions. Spleens were removed aseptically and placed in individual preweighed petri dishes.

Spleen weights were determined and cultured following the procedure of De Ropp (4) with slight modifications. The weighed spleen was placed in a sterile Ten Broek tissue grinder. A volume of 1-percent peptone broth equal to twice the weight of the spleen was added, and the spleen was thoroughly ground. Tenfold dilutions were made of the splenic pulp-peptone mixture, and 0.1 ml. amounts of the various dilutions were inoculated onto the surface of tryptose agar plates enriched with 5-percent bovine serum. These were incubated under 10 percent CO_2 at 37° C. for 5 days at which time colony counts were made.

The extent of splenic infection was approximated by determining the average colony counts of at least two plates. This was for comparative purposes only. De Ropp (4) reported that the probable magnitude of the combined grinding and sampling errors in this procedure was ± 20 percent.

[3] Bausch and Lomb, Inc., Rochester, N.Y.
[4] Atlas Chemical Industries, Inc., Wilmington, Del.
[5] Humble Oil Co.
[6] Abbott Laboratories, North Chicago, Ill.

Results

The results of the 2-month group (table 1) indicate that, under the conditions of this study, strain 45/20 incorporated in adjuvants A and B was ineffective as an immunizing agent. The challenge strain was recovered from 100 percent of the spleens of the guinea pigs receiving these bacterins. The mean spleen counts and seroagglutination titers were equal to or exceeded those of the challenged controls. One guinea pig that received bacterin A intramuscularly was negative (1:12.5 dilution) on the standard-tube test even though the mean spleen count was 2.4×10^3.

The mean values of the other parameters considered varied from group to group. The guinea pigs that received bacterin B intramuscularly had the highest percentage (61.5) with lesions of the vaccinated groups and the highest mean spleen/body weight ratio (0.42) of any of the groups. Both groups that received bacterin B had a mean weight loss following challenge.

Failure to recover the challenge strain from the spleen of two guinea pigs (13.3 percent) receiving bacterin C subcutaneously and one guinea pig (6.6 percent) receiving bacterin C intramuscularly may indicate evidence of an increased resistance of low magnitude in these groups. There was a slight reflection of this in the mean spleen counts. All three guinea pigs that failed to yield the challenge strain on spleen culture were negative (1:12.5 dilution) to the standard tube test. An additiona two guinea pigs (one from each group) were negative to the standard tube test at the same dilution even though their spleen counts were 8.5×10^2 and 3.8×10^5.

The values for the remaining parameters of guinea pigs receiving bacterin C were, with two exceptions, equal to or lower than those recorded for guinea pigs receiving bacterins A and B. The group receiving bacterin A intramuscularly had a slightly lower mean spleen per body weight ratio and the group receiving bacterin A subcutaneously had a higher mean weight gain.

A further increase in resistance was evidenced in the groups receiving bacterin D, in comparison with those receiving bacterins A, B, or C, by a failure to recover the challenge strain from six guinea pigs (42.9 percent) in each group. The mean spleen counts were essentially the same as those observed with bacterin C. Of the 12 guinea pigs that failed to yield the challenge strain on spleen culture, only one was negative (1:12.5 dilution) to the standard tube test. All others had titers ranging between incomplete at the 1:12.5 dilution to incomplete at the 1:200 dilution. Values for the remaining parameters of bacterin D were generally comparable to those observed with bacterin C and generally lower than those observed with bacterin A or B.

The protection afforded guinea pigs by bacterin E appears to be more nearly equal to that of strain 19 than any of the other bacterins evaluated in this study.

The spleens of 10 guinea pigs (66.6 percent) receiving bacterin E failed to yield the challenge strain when cultured as compared to the spleens of 11 guinea pigs (71.4 percent) receiving strain 19. The mean spleen

TABLE 1.–A comparison of the immunizing properties of strain 45/20 bacterins made with various adjuvants and strain 19 living vaccine (2-month group)

Treatment	Route	Mean weight change at challenge	Mean weight change at necropsy	Mean spleen per body wgt.	Mean agglutination titer	Number with lesions[1]	Number with recoveries[1]	Mean spleen counts
		G.	G.	Percent				
Bacterin A	Subcutaneous	134	54	0.28	+1/400	3/13	13/13	6.7×10^5
Bacterin A	Intramuscular	209	19	.22	I1/400	4/13	13/13	5.3×10^5
Bacterin B	Subcutaneous	183	-5	.31	+1/800	6/15	15/15	1.4×10^6
Bacterin B	Intramuscular	204	- 4	.42	I1/600	8/13	13/13	5.9×10^5
Bacterin C	Subcutaneous	173	25	.26	I1/200	2/15	13/15	3.4×10^5
Bacterin C	Intramuscular	164	41	.24	I1/400	4/15	14/15	4.5×10^5
Bacterin D	Subcutaneous	185	37	.24	I1/200	0/14	8/14	4.1×10^5
Bacterin D	Intramuscular	209	24	.24	I1/400	4/14	8/14	4.3×10^5
Bacterin E	Intramuscular	193	52	.16	I1/100	3/15	5/15	5.5×10^4
Strain 19	Subcutaneous	170	55	.15	+ 1/50	1/15	3/14	2.2×10^4
Challenged controls	–	195	13	.35	+1/400	11/14	14/14	5.2×10^5
Unchallenged controls	–	178	80	.13	-1/12.5	0/12	0/12	0

[1] Initially there were 15 guinea pigs per group. Numbers less than this in the denominator indicate the death of guinea pigs during the study in that group.

counts were approximately one log lower for both of these groups. All of the animals from which isolations were made also developed titers. The values for the other parameters in these two groups were lower than those of the other groups, with the exception of the unchallenged controls, which they approximated in most instances.

In reviewing the results of the 7-month group (table 2), it should be borne in mind that the challenge dose (viable cells) was about half that received by the 2-month group.

There was little evidence of increased resistance in any of the groups receiving bacterins A, B, or C. Recoveries were made from the spleens of all guinea pigs receiving bacterin A and bacterin B subcutaneously. The spleen of one guinea pig receiving bacterin B intramuscularly failed to yield the challenge strain on culture; however, the same can be said of the challenge control group that received no treatments before challenge.

The failure to recover the challenge strain from two guinea pigs (16.3 percent) receiving bacterin C subcutaneously may be an indication of a low degree of resistance, but this is not reflected in the other criteria under consideration.

The guinea pigs that were negative on culture were also negative to the standard tube test (1:12.5 dilution). Four additional guinea pigs (two bacterin B subcutaneous, one bacterin C subcutaneous, one bacterin C intramuscular) were negative to the standard tube test at the same dilution but had spleen counts ranging from 6.4×10^1 to 2.0×10^4.

The values of other parameters considered in these groups in general exceeded those of the untreated challenged control group.

Even though the number of challenging organisms was reduced by approximately one-half, all except one group of animals that were treated with bacterins A, B, or C had mean spleen counts that exceeded those with the corresponding treatment in the 2-month group.

With a less severe challenge, bacterin D administered subcutaneously appeared to occupy an intermediate position with regard to its ability to protect guinea pigs against challenge by a virulent strain. When administered intramuscularly, bacterin D appeared to be comparable to strain 19 vaccine and slightly superior to bacterin E. The values observed for the parameters considered in these groups were, in general, lower than those observed with the corresponding treatment in the 2-month group and very nearly equal to those of the unchallenged controls.

Of the parameters considered in evaluating the data obtained from this study, the protection rate (based on spleen recoveries) appeared to be the most meaningful and tended to reflect in the values of other parameters. A direct comparison of these rates for each group is shown in figure 1.

Body weight changes following challenge cannot be equated with the effects of the challenge dose because of the overriding influence of environmental factors. This is particularly true of the 7-month group where considerable increase in size of the individual guinea pigs

TABLE 2.–A comparison of the immunizing properties of strain 45/20 bacterins made with various adjuvants and strain 19 living vaccine (7-month group)

Treatment	Route	Mean weight change at challenge	Mean weight change at necropsy	Mean spleen per body weight	Mean agglutination titer	Number with lesions[1]	Number with recoveries[1]	Mean spleen counts
		G.	G.	Percent				
Bacterin A	Subcutaneous	447	-24	0.24	+400	8/15	15/15	$1x10^6$
Bacterin A	Intramuscular	365	17	.24	+400	8/13	13/13	$6.7x10^5$
Bacterin B	Subcutaneous	333	-14	.22	1800	7/12	12/12	$1.4x10^6$
Bacterin B	Intramuscular	373	- 3	.21	+200	8/14	13/14	$1.9x10^6$
Bacterin C	Subcutaneous	481	-18	.23	+200	6/12	10/12	$6.2x10^5$
Vacterin C	Intramuscular	318	19	.19	1200	3/11	10/11	$8.7x10^5$
Bacterin D	Subcutaneous	374	3	.18	+ 50	2/10	4/10	$2.2x10^5$
Bacterin D	Intramuscular	365	17	.12	+ 50	0/11	0/11	0
Bacterin E	Intramuscular	407	-20	.13	150	0/12	3/12	$6.2x10^4$
Strain 19	Subcutaneous	281	21	.12	+ 25	2/13	1/13	$1.8x10^2$
Challenged controls	–	335	-39	.17	+100	4/12	11/12	$3.6x10^5$
Unchallenged controls	–	359	-50	.13	-12.5	0/12	0/12	0

[1] Initially there were 15 guinea pigs per group. Numbers less than this in the denominator indicate the death of guinea pigs during the study in that group.

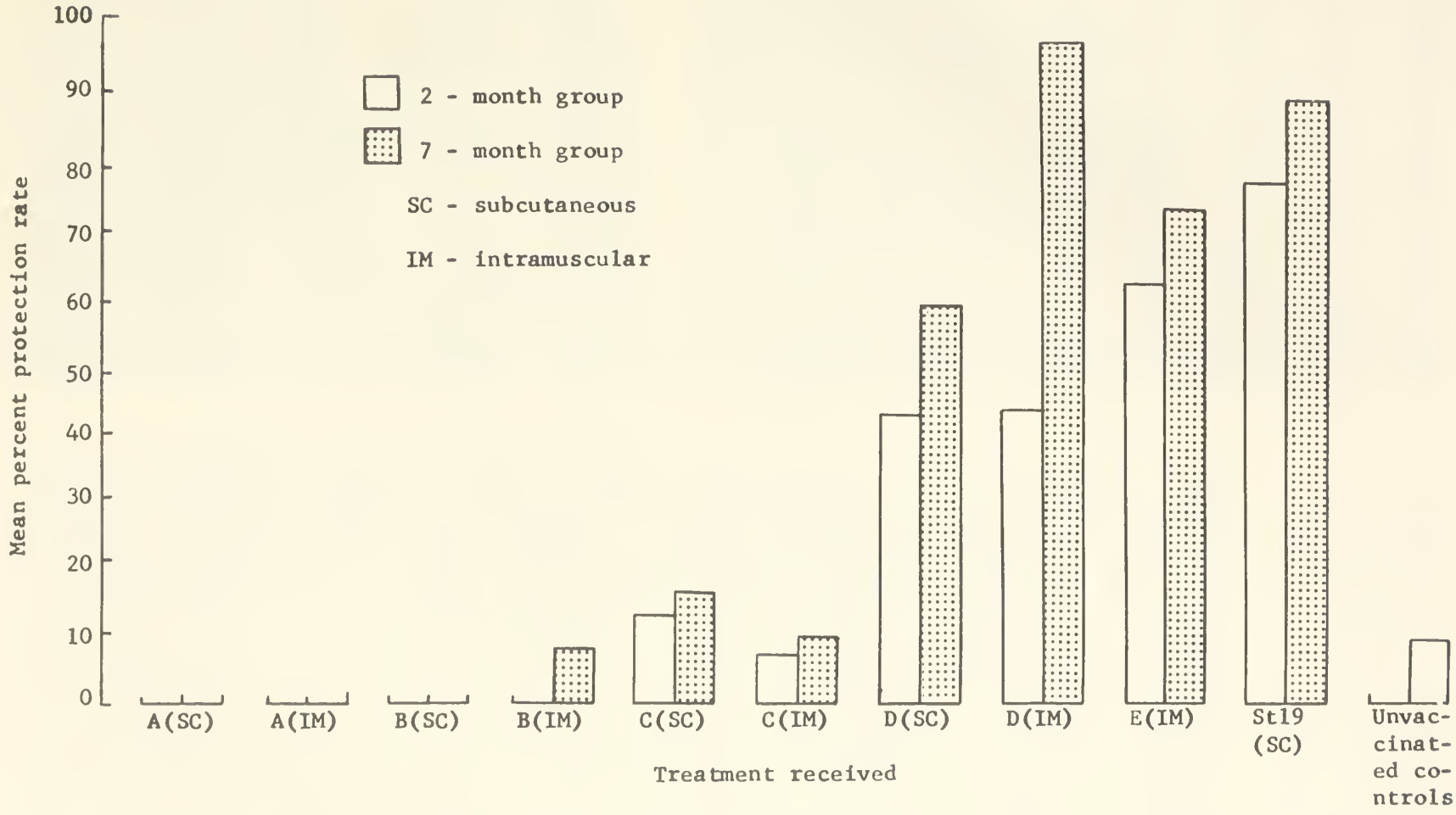

Figure 1.–Mean percent protection afforded guinea pigs by strain 45/20 incorporated in various adjuvants and strain 19 (living) against challenge of a virulent strain (2308) of *Brucella abortus*.

occurred during the period. This led to crowded conditions in individual cages, which was conducive to fighting among the guinea pigs, especially in the instances where a definite peck order was established.

Discussion

The results of this study agree essentially with the findings of McDiarmid and Sutherland (14). They also substantiate the findings of others (11, 12, 13) with regard to the potentiating effects of water in oil emulsions on the immunogenic properties of killed brucella.

Adjuvants A and B were supplied without information as to composition, but the recommendation was to prepare an emulsion by shaking. It was quite evident that there was minimal tissue response following the injection of these adjuvants. Guinea pigs failed to show discomfort from the injections; and when the second injection was administered 2 weeks later, there was no gross evidence of fibrosis or granuloma formation at the initial injection site. At necropsy there was no gross evidence of depot formation at either the subcutaneous or intramuscular sites. This, plus the fact that the suspension prepared from these adjuvants was very unstable (considerable separation occurred upon standing), probably accounts for the failure of these adjuvants to have exhibited potentiating effects.

The tissue response observed with adjuvants D and E were comparable. Following initial injection all guinea pigs exhibited considerable discomfort from the resulting swelling and inflammation. When the second injection was given all guinea pigs evidenced a firm nodular granuloma at the initial injection site. A small granuloma filled with caseous material was evident at necorpsy, particulary at the sucutaneous injection site, in many of the guinea pigs of both groups. It would appear from these observations that the degree of immunity engendered by killed brucella is dependent on the nature of the excipient and directly influenced by the degree of tissue response produced.

It has been stated that probably the major reason for failure to produce immunity against brucella with killed vaccines has been insufficient dosage (18). Perhaps the efficiency of all the bacterins prepared in this laboratory could have been enhanced had the number of killed cells included with the adjuvant been increased. Gwantkin and Dzenis (9) reported a failure to protect guinea pigs with a saline in oil vaccine and attributed it to either a light vaccinating suspension or too severe a challenge. In a subsequent experiment, where the concentration of cells in the adjuvant was increased approximately 130-fold and the challenge does decreased by approximately sixtyfold, a protection rate of 40 percent was demonstrated.

The number of killed cells in bacterin E far exceeded (150-fold) the number in the remaining bacterins used in this study. Yet, when the severity of challenge was decreased, bacterin D (subcutaneous) appeared to be nearly comparable to bacterin E (intramuscular) and superior when bacterin D was administered intramuscularly.

It is interesting to speculate on what the potentiating properties of alginate might have been had the concentration of cells approximated that of bacterin E. Adjuvant C was a preparation of sodium alginate, a polymer of mannuronic acid of controlled polymer length. It has been reported to remain at the site of inoculation for some time giving rise to a foreign body type tissue reaction (1). The observable gross tissue response to this adjuvant was negligible, being comparable to that observed with bacterins A and B. This again probably accounts for the failure of this adjuvant to have significantly potentiated the immunogenic properties of killed strain 45/20. A critical factor, upon which the depoting action of this adjuvant is incumbent, is the availability of soluble Ca^{++} (1). Although this product was pretitrated to reduce the requirement of Ca^{++} supplied by the tissues, it is possible, though highly improbable, that the Ca^{++} available in guinea pig tissues was insufficient to form a coagulum of insoluble calcium alginate.

A representative number of animals from the various vaccinated groups that failed to yield brucella upon culture of the spleen 2 months following challenge were also negative to the standard tube test (1:12.5 dilution).

An additional 7 animals that had a negative serological response were positive to culture (spleen counts ranged from 4.0×10^1 to 3.8×10^5). All of these were in those groups receiving bacterins that failed to produce a significant gross tissue response [A (IM), 1; B (IM), 2; C (SC), 2; C (IM), 2]. Whether this failure to produce detectable agglutinins indicates a relative immune state in these animals is problematical.

Failure of a secondary antibody rise has been described for brucella-infected cattle and was suggested as a means of selecting infected animals (2, 20). Conversely, a marked booster effect has been reported in infected animals with low titers or in the incubation stage following the administration of Duphavac (17). An ability to respond to a secondary antigenic stimulus in the classical fashion has been suggested as an indication of susceptibility to infection in studies on epididymitis in rams (3). Conversely this implies that failure to respond is an indication of an immune state.

It has been shown that single intravenous injection of many protein antigens fail to elicit a measurable immune response in the adult guinea pig (21). It has also been shown that the immune response against certain protein antigens in Freund's adjuvant can be suppressed by intraperitoneal and intradermal injections of the homologous antigen in saline (5). Three steps are usually considered necessary for the induction of experimental unresponsiveness (16). First, antigen is administrered in a nonsensitizing form or by a nonsensitizing route (the suppressing injection). Second, the animal is sensitized with the same antigen, frequently in adjuvant (sensitizing antigen). Finally, after an interval, the immune response is measured and compared with animals receiving only the sensitizing injections. Although the criteria for establishing the unresponsive state does not appear to have been satisfied in these animals, it appears that there was an interference mediated by some undetermined mechanism which prevented the production of classical agglutinins in these animals. It seems unlikely that inherent unresponsiveness would be limited only to these groups. It is conceivable that this phenomenon is an all or none response as has been described for induced unresponsiveness (6). If this same phenomenon occurred in the bovine following the administration of strain 45/20, it could become significant in permitting infected animals to remain undetected.

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Abstracts of Published Reports

Pietz, D. E., Anderson, R. K., Werring, D. F., Kenyon, A. J., and Jenness, R. A modified brucella ring test of cream. Amer. Jour. Vet. Res., 122: 39-44. 1967.

A new method for conducting the brucella ring test (BRT) of cream, named the modified BRT of cream, was compared with the regular BRT of cream. Fresh and stored creams at selected pH levels were used as test samples.

The regular BRT of fresh creams was slightly less sensitive than the BRT of milk, and the sensitivity was reduced further as the pH of the creams decreased during storage.

The modified BRT of fresh creams was equal in sensitivity to the BRT of milk, and the sensitivity was not appreciably affected as the pH of the creams decreased during storage.

In the test of 1,000 cream samples obtained from a State-Federal brucellosis testing laboratory, the modified BRT of cream detected agglutinating antibodies in 3.30 percent of the samples, and the regular BRT of cream detected agglutinating antibodies in only 1.60 percent of the samples.

The modified BRT of cream appears to provide a method of assuring relatively constant sensitivity for detecting brucella agglutinins in cream following storage, regardless of pH changes caused by souring.

Project Reports

Cabrey, E. A., Stewart, W. C., Seaman, E. R., and Rumpler, W. V. (Drs. Seaman and Rumpler are State and Federal hog cholera diagnosticians, respectively, in Ohio.) A perplexing case of hog cholera confirmed by virus isolation and supported by the fluorescent antibody serum neutralization test.

Introduction

The fluorescent antibody, serum neutralization test[1] has been increasingly used as a laboratory aid in confirming the diagnosis of hog cholera (HC). It is now utilized routinely by the HC diagnosticians and will be valuable in the final phases of HC eradication.

The case presented is of particular interest in that the subclinical nature of the infection in some of the pigs in the infected herd caused some doubt in the minds of the field personnel as to the validity of the isolation of HC virus. Since the affected pigs were not vaccinated, it was possible to support the diagnosis by detecting serum antibodies against the infecting virus.

Case Report

On December 29, 1966, two feeder pigs were found dead in a herd of seven sows and 62 feeder pigs. The pigs had been raised on the farm, were not vaccinated for HC, and all had appeared healthy the previous evening. A veterinarian performed necropsies on the dead pigs and diagnosed swine erysipelas. Lesions observed were hemorrhagic lymphadenitis, pneumonia, endocarditis, ascites, petechial hemorrhages of the kidneys, and arthritis. On the following day, the pigs were vaccinated with a formalin-inactivated *Erysipelothrix insidiosa* bacterin. One pig found sick at this time was given additional therapy with antibiotics and anti-erysipelas serum but died 2 days later.

On January 11, 1967, two more pigs were found dead without any history of illness. A different veterinarian was called to the farm and necropsies were performed. Hemorrhages of the lymph nodes and kidneys, gastroenteritis, and cutaneous erythema were observed, which caused the veterinarian to suspect HC. The following day another pig died suddenly and had similar lesions on necropsy.

The State HC diagnostician arrived at the farm on January 12, 1967, and found only one sick pig. The pig had a rectal temperature of 103° F., total white blood cell count of 10,000, slightly incoordinated gait, and fine muscular tremors, but was alert and in good condition. The pig was killed for necropsy and lesions were found as follows: Hemorrhage of the mandibular and suprapharyngeal lymph nodes, small abscesses of the tonsil, pneumona, excess fluid in the pericardial sac, hemorrhage on the epicardium, and edema of the colonic mesentery and the wall of the stomach.

The pigs were housed and placed on a Furacin[2] and water mixture. Specimens of brain in formalin and frozen spleen and tonsil were submitted to the laboratory for examination for HC. Microscopic lesions consistent with a diagnostic of HC were found in the brain and HC virus was isolated by the fluorescent antibody, tissue culture technique: spleen, 1,700 virus plaques per ml. of suspension; and tonsil, 100 plaques per ml. Bacteriologic cultures for Salmonella on Salmonella-Shigella agar[3] and *Erysipelothrix insidiosa* on sodium azide-crystal violet medium were negative.

On January 16, 1967, the HC diagnosticians returned to the farm and found all pigs doing well and apparently healthy. This situation raised some question as to the validity of the diagnosis, and the owner decided not to vaccinate for HC. To confirm or reject the presence of HC virus in the herd, blood samples were collected for serum neutralization tests for HC. Eight samples were collected from the remaining 52 feeders, and three from the seven bred sows which were housed separately from the fat hogs. These serums were examined for antibodies against HC with the fluorescent antibody serum neutralization test.[4] The findings are presented in table 1.

The serum titers of the feeder pigs were quite high and indicated previous infection with HC virus. The absence of antibody titers in two of the three breeding animals housed separately was also significant. The previous isolation of HC virus had been confirmed by the detection of antibody titers in these exposed unvaccinated pigs.

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[1] Lee, L. R., Carbrey, E. A., Kresse, J. I., and Stewart, W. C. Fluorescent antibody, serum neutralization test for detection of hog cholera antibodies employing a plaque reduction technique. *In* Developmental Studies Conducted during Fiscal Year 1966, Diagnostic Services, National Animal Disease Laboratory. U.S. Dept. Agr., Agr. Res. Serv. ARS 91-63 pp. 16-22. 1967.

[2] Eaton Laboratories, Norwich, N.Y.

[3] Difco Laboratories, Inc., Baltimore, Md.

[4] See footnote 1.

TABLE 1.–Serum neutralization titers against HC virus of serums collected Jan. 16, 1967

Pig	Serum titer	
	Dilution	Log_{10}
Feeder	16	1.2
Do	1024	3.0
Do	256	2.4
Do	1024	3.0
Do	256	2.4
Do	1024	3.0
Do	256	2.4
Do	1024	3.0
Bred sow	[1]Neg.	0
Do	Neg.	0
Bred gilt	1024	3.0

[1] Less than 90 percent reduction in the plaque count of the test virus at the 1-16 dilution.

Unfortunately for the owner, this was not the end of his troubles. On March 21, 1967, the diagnosticians were called back to the farm because of death losses in sows and in baby pigs farrowed by these sows, which were pregnant during previous sickness on the farm. One sow and a litter of pigs had died and four of the remaining sows were off feed. Signs of scours and weakness were observed. Tissues from two, 2-week old pigs were submitted to the laboratory and HC virus was isolated from both pigs as follows:

Pig A, spleen - 9,000 plaques/ml.
tonsil - 1,000 plaques/ml.
Pig B, spleen - 2,700 plaques/ml.
tonsil - 1,600 plaques/ml.

Brain lesions consistent with hog cholera were found on histopathologic examination.

To further confirm the diagnosis, a 30 lb. specific-pathogen-free pig was inoculated intramuscularly with 10 ml. of composite tissue suspension from spleens of pigs A and B. The pig became sick and died 15 days postinoculation. Lesions of HC were observed on necropsy and virus was recovered from the spleen, 18 virus plaques per milliliter of spleen suspension.

On May 10, 1967, the HC diagnostician returned to the farm to obtain blood samples from the remaining sows and gilts. Of the seven breeding animals alive in March, only three survived, two had died, and two were sent to slaughter. Samples for serum neutralization tests were submitted to the laboratory.

The antibody titers against HC virus are presented in table 2.

TABLE 2.–Serum neutralization titers against HC virus of serums collected May 10, 1967

Pig	Serum titer	
	Dilution	Log_{10}
Sow - 3798	1024	3.0
Sow - 3799	1024	3.0
Sow - 3800	4096	3.6

Discussion

This case has some unusual aspects in relation to what is expected in a hog cholera infection of susceptible swine. The strain of virus involved was quite lethal in that it killed several pigs in the herd with little or no illness and caused the death of the test pig in 15 days. However, most of the feeder pigs that had developed a subclinical infection recovered and were immune.

The rate of spread from pig to pig was quite slow since the virus persisted in the herd from January 12, 1967, to March 21, 1967, by actual recovery of virus. Of course, the virus could have been carried by the baby pigs in the uterus of one of the pregnant sows during this period of time.

There is a strong probability that other pathogens were active in these pigs and acted together with the HC virus. The response to treatment with the Furacin corroborates this possibility.

The absence of previous vaccination for HC in this herd permitted a clearcut interpretation of the results of the serum neutralization test. As the extent of vaccination with modified-live-virus vaccine is reduced, the serum neutralization test will become the most useful technique to detect the presence of a chronic HC infection in a herd of pigs.

Cabrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. Effect of simultaneous inoculation of pigs with crystal violet, heat inactivated, killed, hog cholera virus vaccine and tissue culture, origin, modified-live-virus vaccine.

Abstract

Twelve pigs were inoculated with crystal violet preserved, killed hog cholera (HC) vaccine 3 days before, the same day, and 3 and 6 days after inoculation with a modified-live-virus vaccine (tissue culture origin). Three

pigs were inoculated with live HC vaccine only for controls. There was no detectable differences in the reactions of these pigs through 18 days after inoculation with live virus. Highest rectal temperatures occurred on the 5th and 6th day in all groups of pigs.

Introduction

The occurrence of hog cholera (HC) in a group of pigs shortly after vaccination with a killed virus (crystal violet preserved, heat-inactivated) vaccine is usually attributed to the coincidental exposure of the pigs to a field strain of HC virus. When the source is obscure, there is a tendency to suspect the presence of viable, infectious virus in the vaccine.

At the time this study was undertaken, incrimination of killed virus vaccines as a source of virulent virus had not been established. To explain some of the outbreaks that had occurred, the following were considered:

a. The inoculation of killed virus antigen produced a negative phase in pigs already exposed to HC.
b. The killed virus antigen in the body of the pigs acted synergistically with a field strain of reduced virulence to produce a severe type of HC.
c. The transformation of an attenuated HC strain to a virulent strain was accomplished by the simultaneous inoculation of a pig with a virulent, inactivated virus and and attenuated, live virus. A basis for this hypothesis was found in the work of Berry and Dedrick[1] who transformed rabbit fibroma virus into virulent myxoma virus by simultaneously inoculating rabbits with live fibroma virus and heat-inactivated myxoma virus.

It was considered of value to inoculate pigs with an attenuated strain of HC virus and, at the same time, to inoculate heat-inactivated antigen from a virulent virus strain to test the validity of the three hypotheses.

Materials and Methods

Pigs.–The pigs used for the experiment were 12 second generation specific-pathogen-free (SPF) pigs and three first generation SPF pigs. The pigs weighed from 35 to 50 pounds.

Virus.–The killed virus (KV) employed as a source of virulent virus was a crystal violet vaccine produced by Southwestern Laboratories, Inc., Wichita, Kans., Serial Number S-56, expiration date Dec. 29, 1968. The live virus (LV) used was a modified-live virus vaccine, Certigen (Muticell Porcine Tissue Culture Origin), Diamond Laboratories, Inc., Des Moines, Iowa, Serial Number 114C, expiration date Dec. 8, 1967.

Protocol.–Twelve pigs were inoculated simultaneously with KV and LV according to the following pattern:

Group A - Three pigs were given 5.0 ml. KV, subcutaneous (s.c.) 3 days before being given 4.0 ml. of LV, intramuscular (i.m.)
Group B - Three pigs were given 5.0 ml. KV, s.c. and 4.0 ml. LV, i.m. on the same day.
Group C - Three pigs were given 2.0 ml. LV, i.m., and then given 5.0 ml. KV, s.c. 3 days later.
Group D - Three pigs were given 4.0 ml. LV, i.m., and then given 5.0 ml. KV, s.c. 6 days later.
Group E - Three pigs were given 4.0 ml. LV, i.m.–this was the LV control group.

Daily observations were made and rectal temperatures were taken

Virus Culture.–Spleen and tonsil specimens were cultured for HC virus on PK-15 coverslip cell cultures and stained with fluorescent antibody conjugate after incubation for 48 hours as previously described.[2]

Serum Neutralizations.–The serums of the pigs were examined for antibodies against HC virus employing the fluorescent antibody, serum neutralization (FASN) test as previously described.[3]

Results

There was essentially no difference observed in the reactions of the pigs in the five groups following inoculation with LV and KV.

The mean daily rectal temperature of the pigs in each group are presented in table 1.

The peak mean temperature of the pigs in the control group receiving only LV occurred on the 5th day following inoculation. The peak mean temperature of the other groups that received KV on different days in

[1] Berry, George P., and Dedrick, Helen M. A method for changing the virus of rabbit fibroma (shope) into that of infectious myxomatosis (Sanarelli). Jour. Bact. 31: 50-51. 1936.

[2] Cabrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. Technical aspects of tissue culture, fluorescent antibody technique. U.S. Livestock San. Assoc. Proc., 69th Ann. Meeting. 1965. pp. 487-500. 1966.

[3] Lee, L. R., Carbrey, E. A., Kresse, J. I., and Stewart, W. C. Fluorescent antibody serum neutralization test for detection of hog cholera antibodies employing a plaque reduction technique. *In* Developmental Studies Conducted During Fiscal Year 1966, Diagnostic Services, National Animal Disease Laboratory. U.S. Dept. Agr., Agr. Res. Serv. ARS 91-63, pp. 16-22. 1967.

TABLE 1.–Mean temperatures of each group of three pigs during the course of the experiment

Date	Group A[1]		Group B[1]		Group C[1]		Group D[1]		Group E[1] (control)	
	Inoculation Schedule	Mean temp. T-100	Inoculation Schedule	Mean temp. T-100	Inoculation Schedule	Mean temp. T-100	Inoculation Schedule	Mean temp. T-100	Inoculation Schedule	Mean temp. T-100
September:										
20	KV	–		–		–		–		–
21		2.5		2.8		2.5		2.2		3.1
22		2.5		2.3		2.3		2.3		2.4
23	LV	1.9	KV,LV	2.4	LV	2.1	LV	2.4	LV	2.4
24		2.2		2.4		2.3		2.4		2.6
25		3.1		3.9		2.9		3.3		2.5
26		2.3		3.3	KV	4.1		3.1		2.2
27		2.3		3.0		3.9		2.9		2.4
28		3.8		3.9		4.7		3.7		4.2
29		4.0		4.1		3.6	KV	2.9		3.0
30		3.3		3.5		3.4		3.1		2.1
October:										
1		3.2		3.5		3.6		2.9		2.3
2		2.9		3.1		2.7		2.7		2.2
3		2.5		2.9		2.7		2.1		2.1
4		2.7		3.5		2.7		2.1		2.2
5		2.5		3.0		2.3		2.3		2.1
6		2.6		2.9		2.1		2.3		2.0

[1] KV, killed virus; LV, live virus Double bracket indicates peak mean temperature.

addition to LV were on either the 5th or 6th day. None of the pigs had a severe reaction which suggested the reactivation of the virulent-killed virus through recombination with the attenuated live virus.

Eighteen days after the inoculation of LV, the pigs were killed and no lesions of HC were found on necropsy. HC virus was not recovered from spleen or tonsil by culture on PK-15 cell cultures employing the fluorescent antibody technique. Antibody titers against HC virus were detected in the serums of all of the pigs given KV and LV (table 2). However, two of the three pigs that received LV alone had no detectable titers at the 1 to 4 dilution.

TABLE 2.–Neutralization titers of serums collected from pigs 18 days after inoculation with live virus

Pig No.	Inoculation[1] schedule	Neutralization titer (Log_{10})
10013	Group E - LV only	[2]Neg.
10014		Neg.
10015		0.6
10005	Group A - KV given	0.6
10006	3 days before LV	2.4
10007		1.2
10001	Group B - KV and LV	0.6
10002	given same day	1.2
10003		1.2
10009	Group C - KV given	0.6
10010	3 days after LV	0.6
10011		0.6
10004	Group D - KV given	1.2
10008	6 days after LV	0.6
10012		1.2

[1] LV, live virus; KV, killed virus.
[2] Less than 90 percent plaque reduction against the test virus (1,000 plaques/ml.) at the 1 to 4 dilution.

Discussion

Reactivation of the virulent virus was not accomplished by simultaneous inoculation of pigs with crystal violet, killed virus vaccine, and modified-live-virus vaccine. On the other hand, no negative phase effect or synergistic action was detected in the four groups of pigs as compared with the control group that received

attenuated live virus only. The limited data obtained by this experiment indicated that the three hypotheses presented in the introduction are not valid.

If virulent HC-virus particles had been created by recombination of genetic material from the killed virus and protein subunits from the attenuated live virus, then the pig involved should have developed acute HC and died. Reculard, and coworkers[4] inoculated pigs with virulent HC virus at 24-hour intervals following inoculation with modified-live-virus vaccine (rabbit origin). The pigs receiving virulent virus up to 3 days after vaccination succumbed to acute HC-infection. However, pigs exposed on the 4th day were resistant and survived. This was attributed to the interference of the attenuated virus.

According to this work then, the most promising group for the detection of virulent virus was Group A. It is interesting to note that one of the pigs in this group had the highest serum neutralization titer, 2.4.

Lee, L. R., Stewart, W. C., Kresse, J. I., and Carbrey, E. A. Isolation and characterization of a porcine enterovirus.

Abstract

The virus was isolated from a tonsil-lymph node pool of a pig suspected of hog cholera infection. Identification was based on cytopathic changes in tissue culture, particle size less than 50 millimicrons, ether resistance, ribonucleic acid core, stabilization by divalent cations ($MgCl_2$), resistance to low pH, morphology on electronmicrograph, and production of specific antiserum in rabbits. Nine swine enteroviruses from field submissions have been isolated and serotyped.

Introduction

Porcine enteroviruses (PE) are widespread in the swine population (1, 3, 4, 8). While the specific pathogenesis of PE has not been determined, they have been associated with poliencephalomyelitis (2), Teschen disease (2, 3), and enteritis (6). At this laboratory PE has been isolated from herds where the clinical diagnosis was hog cholera or transmissible gastroenteritis. It is, therefore, important for the virologist to be able to differentiate between PE and other swine viruses. This report described the isolation and characterization of a PE.

Materials and Methods

A. Herd History

Pigs became sick at 2 weeks of age. They had gummy eyes and temperatures from 101.4° to 103.2° F. On postmortem examination, one pig had "swollen kidneys and plugged ureters." Total white blood cell counts ranged from 7,000-8,000. There was no history of vaccination in the herd. The clinical diagnosis was hog cholera; however, both the fluorescent antibody test by virus isolation on PK-15 cell cultures and histopathologic examination of the brain were negative for hog cholera.

B. Isolation

Tissue Culture Systems.—Primary swine kidney monolayers were used for isolation. The cells were prepared by trypsinizing kidneys from 7-day old pigs and dispersing the cells in Hanks balanced salt solution (BSS) containing 0.5 percent lactalbumin hydrolysate (LAH), 10-percent specific-pathogen-free (SPF) calf serum, and 100 units penicillin, 0.1 mg. streptomycin, 0.2 μg Fungizone[1] and 0.1 mg kanamycin sulfate per ml. The cells were maintained in Earles BSS, plus 0.5 percent LAH, 5-percent SPF calf serum and antibiotics.

Virus Isolation.—The tonsil and lymph node were pooled and processed as described previously.[2] The cells were inoculated, 0.1 ml. per tube, with the supernate and incubated at 37° C.

Virus Purification.—The virus suspension from initial culture was filtered through a 0.3 μ millipore filter. The filtrate was purified by the terminal dilution (T.D.) technique. Three T.D. passages were made and the virus suspension from the last passage was used to propagate virus for characterization studies and antiserum production.

C. Characterization

Particle Size Ultrafiltration.—The virus was filtered through 100 and 50 mμ millipore filters. A

[4] Reculard, P., Sizaret, Ph., and Labert, D. Devenir du virus dans l'Organisme des pores immunises contre la peste classique par les vaccins vivants. Academic Veterinaire de France Bulletin 28, pp. 107-109. 1965.

[1] E. B. Squibb & Sons, Inc., New York, N.Y.

[2] Coverslip tissue culture, fluorescent antibody technique for the detection of hog cholera virus. Protocol, Diagnostic Services, National Animal Disease Laboratory, Ames, Iowa 50010.

sample of each filtrate was titered and this titer compared with a control titer (0.3 μ filtrate).

Ether Sensitivity.—A 20-percent suspension (4.0 ml. virus + 1.0 ml. ethyl ether) was used. The mixture was agitated for 18 hours at 4° C., then poured into a petri plate for 15 to 20 minutes to allow evaporation of the ether. The controls used were an ether-sensitive virus, pseudorabies virus, and an ether-resistant virus, porcine enterovirus.

Metabolic Inhibition.—A solution of 5-iodo deoxyuridine (IuDR), at a concentration of 10 mg. per 100 ml. was prepared in Earles BSS. The nutrient mediums from primary cultures were decanted and replaced by the IuDR solution. After 18 to 24 hours of incubation, the cultures were inoculated with 100, 50-percent tissue culture infective doses ($TCID_{50}$) of virus. Three days after infection the culture mediums were decanted from the cell cultures and titrated for virus activity. The control viruses employed were an enterovirus and pseudorabies virus.

Cationic Stability.—A 2 M solution of $MgCl_2$ was prepared in distilled water and sterilized in the autoclave at 250° F. for 15 minutes. Equal parts of virus and 2 M·$MgCl_2$ were then mixed, incubated in 50° C. water bath for 1 hour, and titrated for virus activity. Appropriate control determinations were made with an enterovirus and pseudorabies virus.

pH Stability.—Tissue culture medium was adjusted to pH 3.0 with 1N HCl. The viruses were diluted 1: 10 in this medium, incubated at room temperature for 5-1/2 hours, and then titrated for virus activity. Appropriate control virus determinations were performed.

Electron Microscopy.—A tissue culture harvest of the virus was prepared in the following manner:

a. Suspension was centrifuged in an International PR-2 for 30 minutes at 2,500 r.p.m.
b. Supernate was filtered through 0.3 μ filter.
c. Filtrate was centrifuged in a Spinco Model L Preparative Ultracentrifuge, #30 rotor, at 30,000 r.p.m. for 30 minutes.
d. Supernate was decanted and centrifuged in a Spinco, #40 rotor, at 40,000 r.p.m. for 150 minutes.
e. Supernate was aspirated from the tubes, tissue culture medium was added to 5 to 10 percent original volume, and pellet was soaked overnight at 4° C.
f. The pellet from step e was resuspended in the tissue culture medium already in tube.
g. The suspension was centrifuged in a SW-39 rotor at 40,000 r.p.m. for 150 minutes.
h. The pellet was examined electronmiscroscopically after mounting in neutralized potassium phosphotungstate negative stain after the method of Brenner and Horne (5).

D. Preparation of Antiserum

Antiserum was prepared in two New Zealand white rabbits. The rabbits were inoculated intramuscularly with a 10 ml. mixture of equal parts virus and Freund's complete adjuvant. Eight 2 ml. booster injections were given intravenously twice weekly. Seven days after the last injection the rabbits were bled out by cardiac puncture, the serum harvested and stored at -20° C. The serum was titered against 100 $TCID_{50}$ of the virus employing the beta method.

Results

The virus was isolated on the second tissue culture passage on primary swine kidney cell cultures (fig. 1). A few foci of rounded cells were detected in 24 hours (fig. 2), and the degeneration progressed with 80 percent or more of the cell monolayer destroyed in 72 hours (figs. 3 and 4). The cytopathic changes were typical of those observed in enterovirus infection.

The results of the characterization studies are presented in table 1.

The results of the characterization studies indicated that the isolate was an RNA virus, smaller than 50 mμ, ether resistant (lack of essential lipids), stabilized by divalent cations ($MgCl_2$), and resistant to low pH. These findings correlated with the group properties of porcine enteroviruses described by Wilner (9).

An electron micrograph of the virion magnified 475,000 times is presented in fig. 5. A suggestion of icosahedral symmetry may be observed. By measuring the magnified virion, the actual size was calculated to be 25 to 30 mμ.

The neutralization titer of the rabbit antiserum against the virus isolate was 3.6.[3] In cross neutralization studies against six enterovirus serotypes, the antiserum neutralized only Enteric Cytopathic Porcine Orphan I (ECPO) virus.[4] The virus isolate was also neutralized by an antiserum prepared against ECPO1 virus.

[3] Titer expressed as the logarithm to the base 10 of the dilution of serum.

[4] Enteric cytopathic porcine orphan 1, 2, 3, 5, 6, and 10 virus kindly supplied by E. H. Bohl, Department of Bacteriology, Ohio State University, Columbus, Ohio.

Figure 1.–Normal swine kidney monolayer. (150X)

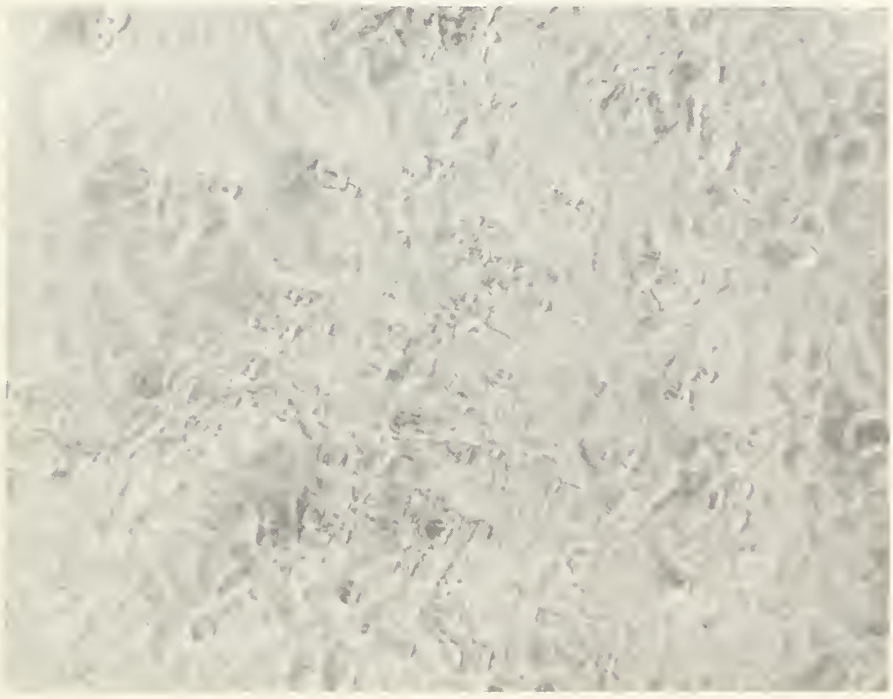

Figure 2.–24 hours–foci developing. (150X)

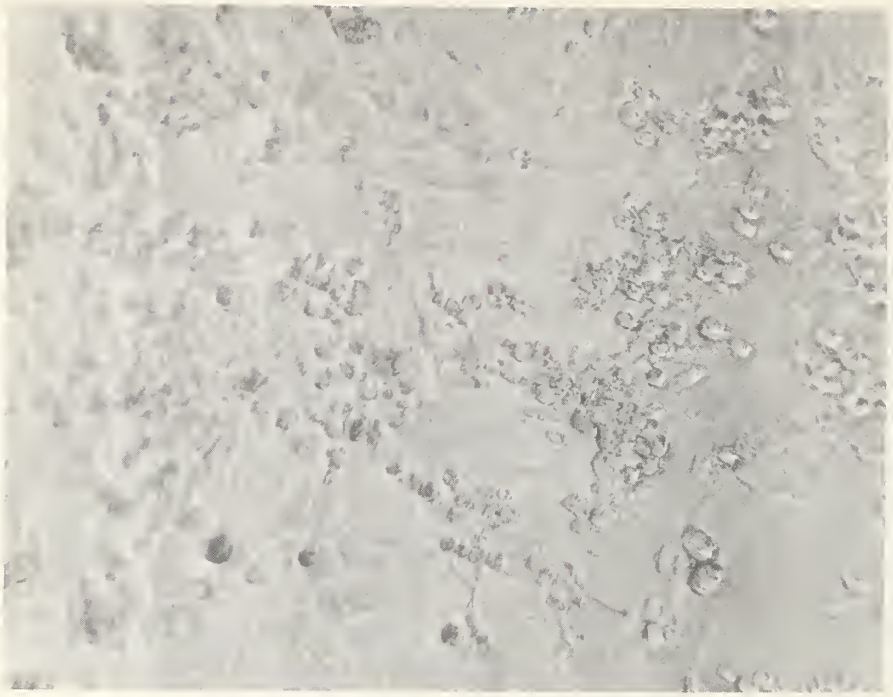

Figure 3.—48 hours—foci spreading through monolayer. (150X)

Figure 4.—72 hours—nearly total destruction of cell monolayer. (150X)

TABLE 1.–Characterization studies on porcine enterovirus isolated from a pig

Test	Variable	Virus titers (Log_{10})		
		Isolate	Aujeszky	Enterovirus
Particle size	Unfiltered	[1]4.0	5.5	–
	100 mμ	3.5	0.0	–
	50 mμ	2.83	0.0	–
Ether sensitivity	Virus only	3.5	5.75	4.5
	Virus + ether	2.75	0.0	3.75
Metabolic inhibition	Virus only	4.5	5.5	5.5
	Virus + IuDR	4.83	1.16	5.5
Cationic stability	Virus only - no heat	4.16	4.83	5.5
	Virus + $MgCl_2$ + heat	4.83	0.0	4.5
pH stability	Virus + pH 7.5	3.5	5.5	4.83
	Virus + pH 3.0	3.5	0.0	5.16

[1] The titers were calculated by the method of Karber (7).

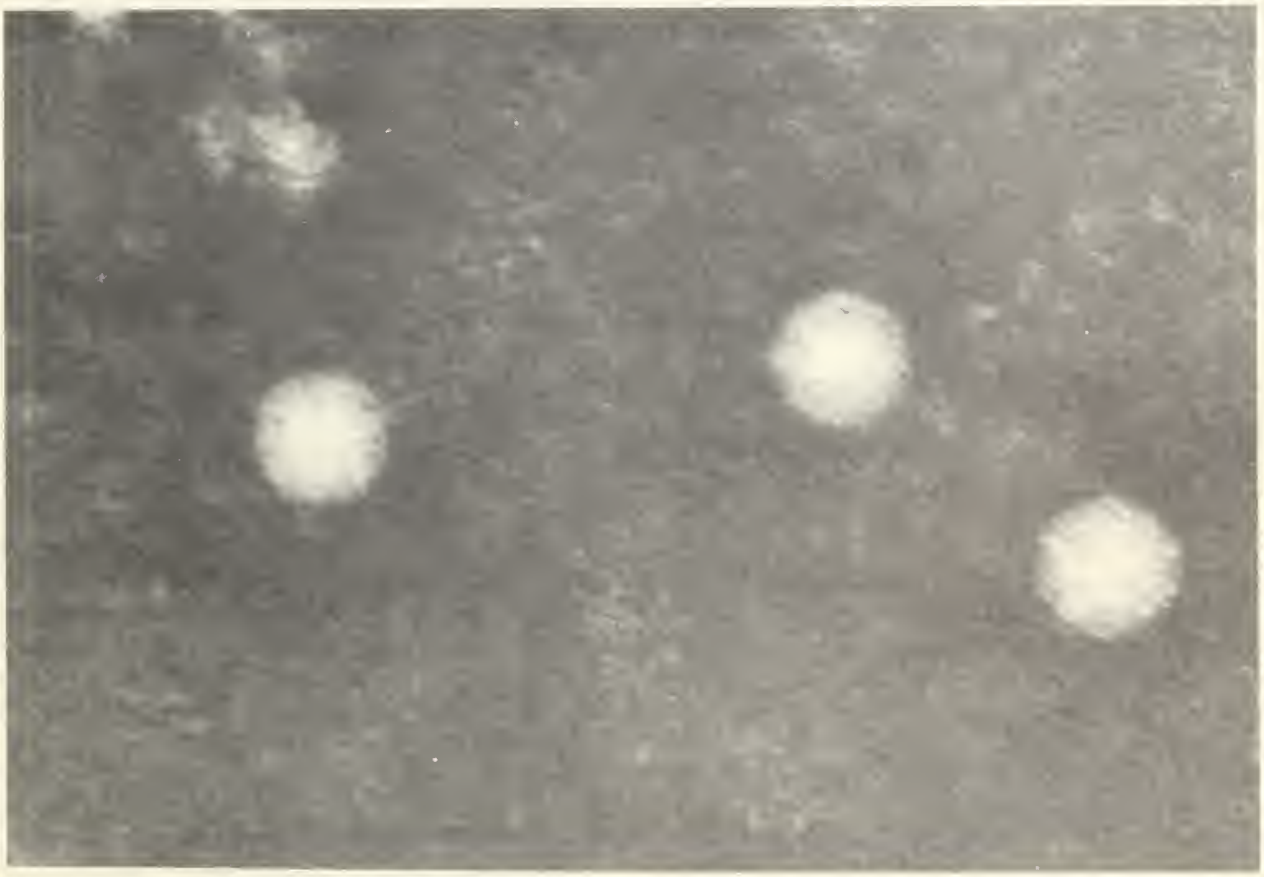

Figure 5.–Electron micrograph of porcine enterovirus. (800,000X)

Discussion

The cytopathic changes produced in tissue culture, the characterization studies, the electron micrograph, and the serum neutralization results all correlate in identifying the ioslate as an enterovirus. The cross neutralization tests demonstrated that there is sufficient immunological differences between the serotypes to accurately identify them in this manner.

Characterization was of value not only in identifying the isolate but also in providing us with a thoroughly characterized enterovirus strain of field origin. To expand our diagnostic capabilities to include identification of swine enteroviruses, there was a clear need for the preparation of antiserums against the frequently encountered strains. We currently have prepared antiserums against six serotypes.

To date nine swine enteroviruses have been isolated from field submissions. Eight of the isolations have been made from tonsils and tonsil-lymph-node pools and one from both the spleen and tonsil-lymph node. The serotypes of the nine were as follows: six ECPO 6 strains, two ECPO 1, and one ECPO 2. A tenth isolate, with typical cytopathic changes, was not neutralized by any of the six antiserums and is currently being characterized.

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(3) Betts, A. O., Kelly, D. F., Lamont, P. H., and Sheffy, B. E. 1961. The isolation and characterization of some enteroviruses from pigs. Vet. Record. 73(31): 752-754.

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Cassidy, D. R., and Seberg, S. A. Studies of serum enzymes in swine infected by two strains of hog cholera virus.

Abstract

Levels of five serum enzymes were determined in 196 swine blood samples. An elevation of lactic dehydrogenase (LDH) and serum glutamic-oxaloacetic transaminase (SGOT) was detected in the sera from swine infected with virulent hog cholera virus. Changes in these two parameters were not detected in swine infected with a strain of hog cholera virus of low virulence. Preliminary findings indicate that porcine serum acid phosphatase, alkaline phosphatase, serum glutamic oxaloacetic transaminase, and serum glutamic pyruvic transaminase (SGPT) are stable in solidly frozen swine serum for at least 5 days. Porcine serum lactic dehydrogenase is apparently inactivated by freezing.

Materials and Methods

Enzyme Assay Procedures.—All serum enzyme assays were performed using commercial reagent kits.[1] The serum was separated by centrifugation, cooled at 24° C.

[1] Sigma Chemical Co., 3500 DeKalb St., St. Louis, Mo.-63118, (SGOT & SGPT were determined by Sigma Method No. 500; LDH by Sigma Method 505; and Acid and Alkaline Phosphatase by Sigma Method 104).

for 1 hour, and assayed for the five enzymes. Stabilized human serum[2] was assayed concurrently to monitor the accuracy of the assays.

Animals

Phase I.–Six, 30 lb., specific pathogen-free (SPF) pigs, fed a nutritionally complete ration and maintained on concrete floored animal shelters were used. Two animals were randomly allotted as controls. Before and after infection body temperatures, levels of serum SGOT, SGPT, LDH, acid, and alkaline phosphatase were determined and recorded on a daily basis. The animals were infected by the intramuscular infection of 2 ml. of hog cholera infected blood. The hog cholera virus was the Ames strain (SN-325), which has been previously passed in pigs 325 times. In the past it has generally killed 30 to 40 pound pigs in approximately 5 days.

All animals that died or were euthanized at the end of 1 month (postinoculation) were subjected to complete postmortem examination. The spleen tissues were examined for hog cholera virus using the fluorescent antibody tissue culture technique. The brains of all animals were examined by routine histologic methods for evidence of encephalitis.

Phase II.–Procedures and conditions similar to those described under Phase 1 were used. The major dfference was that a hog cholera virus of low virulence was used. Total white blood cell numbers were determined as frequently as time permitted. After 1 month, the study was terminated.

Phase III.–An amount of blood sufficient to supply 30 ml. of serum was collected from each of five normal, 50 pound pigs. The serum was separated by centrifugation and divided into five vials containing 5 ml. each. The tubes of serum from each animal were frozen in a deep freeze at -51° C. (72° F.). The levels of serum glutamic pyruvic transaminase, acid phosphatase, and alkaline phosphatase were determined immediately on the one tube of serum from each animal. On each of the next 5 days, the same enzyme levels were determined on one sample of frozen serum from each of the six animals. The adverse effects of freezing on the lactic dehydrogenases in swine serum were observed during the two earlier phases, consequently assays for this enzyme were not performed.

When hemolyzed blood samples were received, they were discarded as abnormally elevated levels and other erratic results were obtained in several previous assay series.

[2] Hyland, Div. Travenol Laboratories, Inc., Los Angeles, Calif.

Results

Phase I.– The levels of both SGOT and LDH were elevated in the serum of swine following their infection with virulent hog cholera virus. In table 1 are listed the means and ranges of the five serum enzymes obtained before and after inoculation with virulent hog cholera virus. Serum enzyme levels were determined daily for 2 weeks before infection. Assays of the serum enzymes were carried out daily after infection until the animals died. All experimental animals died from 3 to 10 days following inoculation.

Hog cholera virus was demonstrated in tissues from the infected animals by the fluorescent antibody tissue culture technique (FATCT). Lesions of encephalitis were demonstrated histologically in the brains of the infected animals but not in the controls. Total white blood cell numbers remained at normal levels in the control animals; however, marked leukopenias were observed in the infected animals.

The assay of hemolyzed blood consistently produced erratically elevated findings with serveral enzymes.

Phase II.–No significant changes were observed in the serum enzyme levels of the animals infected with the hog cholera virus of low virulence (table 2). Transient leukopenias were observed in the infected animals and their body temperatures were elevated following infection. The FATCT failed to detect hog cholera virus in tissues from the infected animals. In two of the inoculated animals, encephalitic lesions were observed histologically. The changes present in the brains of the remaining two infected animals were questionable.

Phase III.–The lack of effects of freezing on swine serum SGOT, SGPT, acid phosphatase, and alkaline phosphatase are evident from the results recorded in table 3.

Discussion

The serum enzymes have been used for the clinical diagnosis of many diseases in both man and animals. Serum glutamic oxaloacetic transaminase (SGOT) is an enzyme that regulates the reversible transamination between alpha-ketoglutaric acid on aspartic acid on one hand, and oxaloacetic and glutamic acid on the other. Oxaloacetate forms oxidized diphosphopyridine nucleotide and l-malic acid in presence of reduced diphosphopyridine nucleotide. Serum glutamic pyruvic transaminase (SGPT) levels are determined on the basis of this enzyme's ability to catalyze the reaction between alpha-ketoglutarate and laevo-alanine to produce glutamate and pyruvate. The pyruvate in the presence of

Table 1.–Effect of virulent hog cholera on serum enzymes–Phase I

Serum enzymes	Animal No.	Mean and range	
		Preinoculation	Postinoculation
Serum glutamic-oxaloacetic transaminase (SGOT)	[1] 10773	27.0(10-48)	23.0(10-42)
	[1] 10774	28.9(20-36)	28.3(19-40)
	10775	28.8(18-44)	74.5(32-166)
	10776	30.6(16-50)	51.6(18-110)
	10777	20.4(12-28)	59.0(20-140)
	10778	27.7(20-36)	48.6(28-82)
Serum glutamic pyruvic transaminase (SGPT)	[1] 10773	23.4(20-32)	28.3(17-50)
	[1] 10774	22.8(18.5-32)	32.8(24-42)
	10775	23.0(19-36)	31.5(28-34)
	10776	25.1(18.5-33)	24.2(20-32)
	10777	20.9(12-28)	29.1(18-49)
	10778	33.5(20-36)	39.3(32-44)
Lactic dehydrogenase (LDH)	[1] 10773	959.4(540-1560)	2545.0(1160-3500)
	[1] 10774	1485.0(1000-3200)	2077.1(1120-2900)
	10775	1517.7(1000-3700)	4800.0(1680-10,000)
	10776	871.0(60-1180)	2824.0(1720-4200)
	10777	947.5(700-1180)	4764.0(1980-10,000)
	10778	1239.0(940-1640)	5360.0(1720-9000)
Alkaline phosphatase	[1] 10773	7.0(4.0-12.2)	6.50(4.8-8.1)
	[1] 10774	8.06(5.2-10.2)	9.00(5.7-12.4)
	10775	4.30(3.0-7.2)	4.10(2.8-5.4)
	10776	5.39(3.23-10)	3.15(1.2-6.4)
	10777	5.50(3.0-10.6)	2.75(1.0-4.5)
	10778	7.98(3.4-9.2)	8.06(4.2-10.9)
Acid phosphatase	[1] 10773	1.75(1.7-2.05)	1.85(1.4-2.25)
	[1] 10774	1.80(0.90-2.65)	1.90(1.3-2.4)
	10775	1.52(3.0-7.2)	1.70(2.8-5.4)
	10776	1.78(0.85-2.45)	2.46(1.4-3.5)
	10777	1.90(1.0-3.4)	2.30(1.35-3.3)
	10778	2.50(2.25-2.85)	1.96(1.4-2.25)

[1] Control animals.

Table 2.–Effect of infection by hog cholera virus of low virulence on porcine serum enzymes–Phase II

Serum enzymes	Animal No.	Mean and range	
		Preinoculation	Postinoculation
Serum glutamic-oxalo-acetic transaminase (SGOT)	63	21.0(10-33)	22.0(8-92)
	64	20.8(2-29.5)	18.3(4-42)
	65	22.6(14-29.5)	18.1(7.0-28)
	66	24.4(10-42)	11.6(4-42)
	[1]67	18.8(3-28)	16.6(2-42)
	[1]68	25.8(16-38)	27.8(12-43)
Serum glutamic pyruvic transaminase (SGPT)	63	21.0(12-28)	17.2(12-24)
	64	30.7(20-58)	20.8(14-32)
	65	19.6(16-26)	20.8(14-32)
	66	24.4(12-42)	24.5(20-33)
	[1]67	29.2(16.3-49)	30.3(24-36)
	[1]68	26.8(16-56)	25.3(20-32)
Lactic dehydrogenase (LDH)	63	1072(840-1360)	1787(350-5100)
	64	1276(1020-1700)	2343(800-5000)
	65	1413(1080-2000)	1722(800-5200)
	66	1442(100-2000)	1663(350-2900)
	[1]67	1184(80-1740)	1523(550-2300)
	[1]68	1520(960-2000)	2076(900-4200)
Alkaline phosphatase	63	5.4(3.7-8.4)	5.4(2.8-6.8)
	64	4.4(2.9-5.6)	4.3(1.9-6.4)
	65	5.5(3.8-7.4)	6.5(1.4-10.2)
	66	4.2(3.2-5.5)	5.1(1.5-6.8)
	[1]67	6.8(6.0-9.3)	5.8(4.8-7.6)
	[1]68	7.2(4.8-10.8)	5.8(0.85-10.4)
Acid phosphatase	63	1.4(0.95-1.7)	1.8(0.60-2.05)
	64	1.3(1.2-1.5)	1.8(1.5-2.35)
	65	1.3(0.60-2.15)	1.9(1.6-2.3)
	66	1.6(1.3-1.7)	1.6(1.3-2.05)
	[1]67	2.0(1.4-2.2)	2.2(1.4-3.0)
	[1]68	2.0(1.9-2.3)	2.2(1.06-2.8)

[1]Control animals.

TABLE 3.–Stability of porcine enzymes when frozen–Phase III

Day	mean range observed in animals			
	SGOT[1]	SGPT[1]	Acid phosphatase	Alkaline phosphatase
1[2]	24.5 (18-26)	26.8 (22-32)	2.16 (1.75-2.45)	5.86 (5.0-6.6)
2	18.8 (22-32)	27.5 (22-32)	2.14 (1.55-2.90)	6.93 (6.6-7.2)
3	29.0 (22-36)	18.3 (15.9-22)	2.07 (1.90-2.40)	7.45 (6.9-8.0)
4	22.1 (20-28)	25.0 (20-32)	2.03 (1.6-2.35)	6.98 (5.8-7.6)
5	38.8 (19.5-58)	24.0 (22-26)	2.62 (2.05-3.10)	8.80 (6.8-11.2)

[1] SGOT–serum glutamic - oxaloacetic transaminage; SGPT–serum glutamic pyruvic transaminase.
[2] Day serum was collected.

reduced diphosphopyridine nucleotide (DPN) is then reduced to lactate and oxidized DPN. The level of the several lactic dehydrogenase isozymes in serum which are linked to DPN, is based on their ability to convert an anaerobic product of carbohydrate metabolism, lactate, to pyruvate. Pyruvate is produced in the Kreds' tricarboxylic acid cycle. Normal blood serum contains several enzymes or groups of enzymes which catalyze the liberations of inorganic phosphate from phosphate esters, such as glycerophosphate and phenylphosphate. The two groups of enzymes are active at pH optima of 9 and 5. The most active phosphatase and one longest recognized is most active at a pH of around 9 and is known as "alkaline phosphatase." A second phosphatase called "acid phosphatase" is most active around a pH of 5.

These results indicate that levels of SGOT and LDH in swine are elevated when the animals are infected by virulent strains of hog cholera. The enzyme levels frequently reach their peak just before death of the animal. Detectable changes were not present in the serum of animlas infected with strains of hog cholera virus of low virulence.

Assays of SGOT, SGPT, acid, and alkaline phosphatase on serum frozen solidly for 5 days suggest that the enzymes are not affected significantly by freezing. The possible utilization of enzyme assay for diagnostic assistance in field problems is suggested by these preliminary findings.

Lee, L. R., Carbrey, E. A., Stewart, W. C., and Dresse, J. I. Susceptibility of embryonic swine spleen and swine kidney cell lines to hog cholera virus.

Abstract

Two tissue culture cell lines, embryonic swine kidney and embryonic swine spleen, were established and compared with the PK-15 swine kidney cell line for susceptibility to hog cholera virus. The two new cell cultures were less sensitive to virus infection than the PK-15 cell line.

Introduction

The PK-15 cell line is used routinely for the isolation and identification of hog cholera (HC) virus employing a fluorescent antibody conjugate to detect virus-infected cells. Difficulties in isolating some field strains of hog cholera virus have occurred. It was considered of value to establish some additional swine cell lines and compare the growth of HC virus in these lines with that obtained with the PK-15 cell cultures.

Materials and Methods

Cell Lines.–The PK-15, pig kidney cell line was obtained from Cutter Laboratories, Inc.[1] The swine kidney and spleen lines were established from the tissues of a pig fetus obtained at a slaughter plant. The PK-15 cell culture was propagated from a single cell to establish a uniform line, while the spleen and kidney lines were propagated in continuous passage from the original tissues. All three lines were grown in modified Eagles medium containing nonessential amino acids and L-Glutamine,[2] supplemented with lactalbumin hydrolysate (25 g. per 5 l.), sodium pyruvate[3] (50 ml. per 5 l.), antibiotics and 10 percent specific-pathogen-free (SPF) calf serum. Leighton tubes with coverslips were prepared as described previously.[4] The spleen and kidney cell cultures were utilized from the 11th to the 14th passages.

[1] Cutter Laboratories, Inc., 4th and Parker Streets, Berkeley, Calif.

[2] Grand Island Biological Co. (GIB), 3175 Staley Road, Grand Island, N.Y. 14072.

[3] 100X, G.I.B. Cat. No. 136.

[4] U.S. Agricultural Research Service. Coverslip tissue culture, fluorescent antibody technique for the detection of hog cholera virus. U.S. Dept. Agr., Agr. Res. Serv. pp. (Processed.) 1964. Diagnostic Services, National Animal Disease Laboratory, Ames, Iowa.

Inoculation.–Cell cultures were inoculated with tissue culture adapted, HC endpoint virus[5] when confluent. The virus suspension was diluted 1:1000 in culture medium. Coverslips were stained, examined by fluorescence microscopy, and virus plaque counts were made.

Results

The swine spleen cell line grew slowly and failed to produce confluent cell cultures in less than 7 days. The susceptibility of this cell culture to HC virus was quite low and a nonspecific, granular fluorescence was observed throughout the cell sheet after the application of conjugate. The swine kidney cell line grew more slowly than the PK-15 cell line, but confluent cultures were produced in 7 days.

The mean virus plaque counts of the PK-15 cell cultures were consistently higher than those of the swine kidney cell line in a series of three trials (table 1).

TABLE 1.–Mean virus plaques per ml. of virus suspension obtained with PK - 15 and swine kidney cell cultures

Trial No.	PK - 15		Swine kidney	
	Age[1]	Plaque counts	Age	Plaque counts
1	2	2,140	7	1,780
2	3	8,700	6	5,130
3	2	2,140	8	710
Mean	2.33	4,327	7	2,540

[1] Age of cells in days at time of inoculation.

Discussion

In this study the PK-15 cell line was more susceptible than the swine kidney and spleen cell lines. This was similar to the findings obtained in a comparison of susceptibility between the PK-15 cell line and primary pig kidney cells reported previously.[6]

One factor contributing to a lowered susceptibility was the number of days required for the swine kidney and spleen cell lines to become confluent. It has been observed with the PK-15 cell line that susceptibility decreases as the age of the cell culture increases.

The rapid growth and uniformity of the PK-15 cell line made it more desirable for virus culture than the two cell lines that were produced in our laboratory.

[5] Lee, L. R., Cabrey, E. A., Kresse, J. I., and Stewart, W. C. Fluorescent antibody, serum neutralization test for detection of hog cholera antibodies employing a plaque reduction technique. *In* Developmental Studies Conducted Furing Fiscal Year 1966, Diagnostic Services, National Animal Disease Laboratory. U.S. Dept. Agr., Agr. Res. Serv. ARS 91-63, pp. 16-22, 1967.

Mengeling, W. L. Pirtle, E. C., and Torry, J. P. Identification of Hog Cholera Viral Antigen by Immunofluorescence. Application as a Diagnostic and Assay Method. Canada Jour. Comp. Med. and Vet. Sci. 27: 249-252. 1963.

[6] Cabrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. Technical aspects of tissue culture fluorescent antibody technique. U.S. Livestock San. Assoc. Proc. 69th Ann. Meeting. 1965. pp. 487-500. 1966.

Cassidy, D. R., Ringham, C. K., and Teig, K. O. The estimation of swine blood leukocyte numbers by the DNA viscosity test.

Abstract

A relatively accurate, economical method for estimating total white blood cell numbers and its application to the enumeration of swine blood leukocytes is described. A high degree of correlation between total leukocyte counts determined by this procedure and the hemacytometer–microscope method was obtained with blood from both normal and hog cholera-infected pigs. Field studies in six States by hog cholera diagnosticians demonstrated an agreement of approximately 90 percent between the two methods.

Literature Review

The physical-chemical gelation, reaction between surface-active chemicals and cells was first observed in milk and described by Schalm and Noorlander (10) in 1957. The method utilized the surfactant, alkyl aryl sulfonate, and was known as the California Mastitis Test (C.M.T.). Jaartsveld (4) in 1961 published a modification of the C.M.T. which involved measuring the time required for a standard volume of milk-reagent mixture to flow through a calibrated capillary funnel. Schalm and Murray (9) in 1964 published the first description of this procedure modified to permit its use in estimating blood leukocyte numbers. The role of deoxyribonucleic acid (DNA) in this gelation reaction resulting from the interaction of a surfactant and cell nuclear deoxyribonucleic acid has been confirmed by Carroll and Schalm (3), who consistently prevented the reaction or produced almost instantaneous dissolution of the reaction-product-gel by the addition of 1.65 μg. deoxyribonuclease per milliliter of mastitic milk. Paape, Snyder, and Hafs (6) confirmed these findings with deoxyribonuclease and also correlated the C.M.T. scores with DNA Feulgen reaction scores on the same milk.

The correlation coefficient was 0.936 (P 0.01). Later these same workers (7) compared six methods for estimating somatic cells in milk and confirmed their earlier results with the Feulgen-DNA test by assaying the results with reflectance spectrophotometry. Vendrely (12) cited results strongly suggesting the the DNA content of the mammalian cell nucleus is a constant characteristic of a species. Recently, Carper and Mitten (2) described a modification of the DNA viscosity test designed to stablize the procedure and eliminate ciritical time factors in the procedure. Morgan (5), after extensive comparative studies between the DNA viscosity method and standard and unopette hemacytometer procedures, reported the DNA method as accurate as any available if the exact technique is used.

Materials and Methods

The principle of the DNA viscosity procedure consists of determining the time for a measured volume of reagent (3 ml.) and one drop of blood to flow through the calibrated stem of a capillary funnel. Theoretically the viscosity of the mixture is proportional to the amount of DNA from cell nuclei in the drop of blood. The constancy of the content of DNA in the somatic cell nuclei should justify the assumption that the viscosity of the mixture is proportional to the number of cells in the drop of blood. An additional assumption is that all of the DNA in the peripheral blood is found in the leukocytes. Duration of the reaction between the blood and reagent and the extent of mixing appear to affect the viscosity.

Flow Time Determinations:

1. Three ml. of reagent were dispensed into a graduated contrifuge tube.

2. One full drop of well-mixed swine blood was added, a stopwatch was started, and the blood and reagent were mixed by inverting the tube slowly *exactly* five times.

3. The funnel stem was pressed onto a rubber stopper and at 20 seconds from adding the blood, the reagent-blood mixture was poured into the funnel.

4. Exactly 30 seconds after adding the blood, the funnel was raised and the stop watch returned to zero and started again. When the fluid level had fallen to the level of the top of the funnel stem, the watch was stopped and the elapsed time recorded. As stated previously, a mean of three flow times was obtained. The mean flow times and hemacytometer counts were used in developing a conversion table (table 1).

5. Flow times of less than 6 seconds indicated a low number of leukocytes. In these cases two drops of blood were used in the test. No increase in flow time indicated a leukopenia. The resulting flow time from two drops of blood divided by two gave the estimated number of leukocytes in the sample of blood.

6. When leukocyte numbers were high enough to produce a flow time of over 25 seconds, one drop of blood was added to 6 ml. of reagent and the flow time determined in two funnels. The results were multiplied by two.

White blood cell numbers were determined on all swine blood samples by the hemacytometer method. All samples were counted by two experienced technicians and the results averaged. When a mean difference in excess of 20 percent between counts was obtained, the

TABLE 1.–Conversion of capillary funnel flow-times to estimated leukocyte numbers/cu. mm. of blood

Flow-time in seconds	Mean total leukocyte number/cu. mm.				
	Dog[1]	Pig	Horse[1]	Cow[1]	Cat[1]
5	–	4,200	–	–	5,000
6	7,500	6,800	5,500	5,500	10,000
7	12,000	9,400	8,500	8,500	14,000
8	15,000	11,600	11,000	11,000	17,000
9	17,500	13,600	13,500	13,500	20,000
10	20,000	15,400	15,000	15,000	23,000
11	21,500	17,000	16,000	16,500	26,000
12	23,500	18,600	17,000	18,000	28,000
13	25,000	20,200	18,000	18,500	30,000
14	26,000	21,600	19,000	19,500	32,000
15	27,500	22,800	20,000	20,000	35,000
16	29,000	24,000	21,000	21,000	–
17	30,000	25,000	22,000	21,500	–
18	31,500	26,000	23,000	22,000	–
19	33,000	–	24,000	23,000	–
20	34,000	–	25,000	23,500	–
21	35,500	–	26,000	24,500	–
22	37,000	–	27,000	25,000	–
23	38,000	–	28,000	26,000	–
24	39,000	–	29,000	26,500	–
25	40,500	–	30,000	27,000	–

[1] Data on the dog, horse, and cow were supplied by O. W. Schalm (9). Information on cat blood was received from O. W. Schalm, October 6, 1967.

sample was recounted by both technicians. A modification of Schalm's DNA viscosity test was used to estimate the number of leukocytes in each blood sample. The arithmetic mean of three tests were recorded for each sample.

The equipment and reagents utilized in the procedure were similar to those used by Schalm and Murray (9).

Funnels.—The funnel[1] is glass and of special design, with a stem 2.0 cm. in length with a 1.3 mm. bore. The funnel capacity is 4.5 ml. The flow time for each of 53 funnels was obtained by determining the time required for 3.0 ml. of the reagent to flow through each funnel. The flow time for each funnel was determined three times.

Reagent.—A commercially available reagent[1] containing 3-percent anionic surface active agent and brom cresol purple at a 1:1000 concentration and neutral pH was used.

Reagent Thermostability.—Because the reagent when in use would be subjected to temperature extremes while in the field, the following study was carried out.

1. Three, 8-ounce polyethylene bottles were filled with reagent and the individual bottles subjected to the following treatments:

   Control - 17 hours at refrigerator temperature of 4° C. (30.2° F.).
   Heated - 17 hours at 70° C. (158° F.).
   Frozen - 17 hours at -51° C (-72.4° F.).

2. Ten samples of swin blood were then tested, using the three samples of reagent as soon as they had reached room temperature 24.4° C. (75.9° F.).

The results were subjected to analysis by the use of a completely random block design with subsampling. Temperatures were considered as treatments and animals as experimental blocks.

Blood.—Heparinized swine blood samples from animals of all age ranges and from both normal and hog cholera-infected swine were examined. In most instances flow times were determined and hemacytometer counts made on the blood the same day it was collected. In a few instances the blood was held overnight at refrigerator temperatures. Serum pipettes were used in place of regular eye droppers used by Schalm (9).

Animals.—The samples were obtained from three groups of swine.

Group I. This group included 195 blood samples from pigs of both sexes, varying in age from 6 weeks to 1 year. It included those in both of the other groups.

Group II. The 20 animals in this group were normal pigs approximately 3months of age. The blood samples were collected 1 day before inoculation with virulent hog cholera virus.

Group III. These 14 animals were survivors of Group II. The blood samples were collected 4 days after inoculation with virulent hog cholera virus.

The resulting data are subjected to appropriate statistical treatment and then plotted by a computer.

Field Studies

Six hog cholera veterinary diagnosticians from Missouri, Kentucky, Ohio, Oklahoma, Nebraska, and Illinois were instructed in the use of the DNA viscosity test. They were furnished with the essential equipment and instructed to submit hemacytometer counts and DNA viscosity flow times on swine blood samples collected during their diagnostic activities. The flow times were interpolated to corresponding hemacytometer values by means of the previously determined conversion data sheet. Comparison of the values was undertaken by means of an analysis of variance.

Results

Flow Time Determinations and Animals.—The results of these determinations and their relationship to the corresponding hemacytometer counts are plotted on the graph shown in figure 1. (Plot of all three groups; Schalm's plots; Carper's plots.) The relationship between funnel flow times and leukocyte numbers of swine blood from three groups of swine can be seen in the graph.

Statistical treatment of the three groups of data was undertaken. A regression coefficient, regression equation, and correlation coefficient were calculated for each of the three groups of data and then plotted. All calculations and plotting were performed at the Iowa State University Computational Center.

Group	*Regression equation*	*Correlation coefficient*
1	Y=3,535.8+1,195.2X	0.83
2	Y=1,147.6+1,141.2X	.89
3	Y=11,548.2-11,548.2	.86

Funnels.—The mean flow time for the 53 funnels was 4.6 ± 0.48 seconds (standard deviation). A 95-percent confident interval was 4.12 to 5.08 seconds. (This means

[1] Funnels and reagents are available from Dairy Research Products, Inc., Harmouth, Maine.

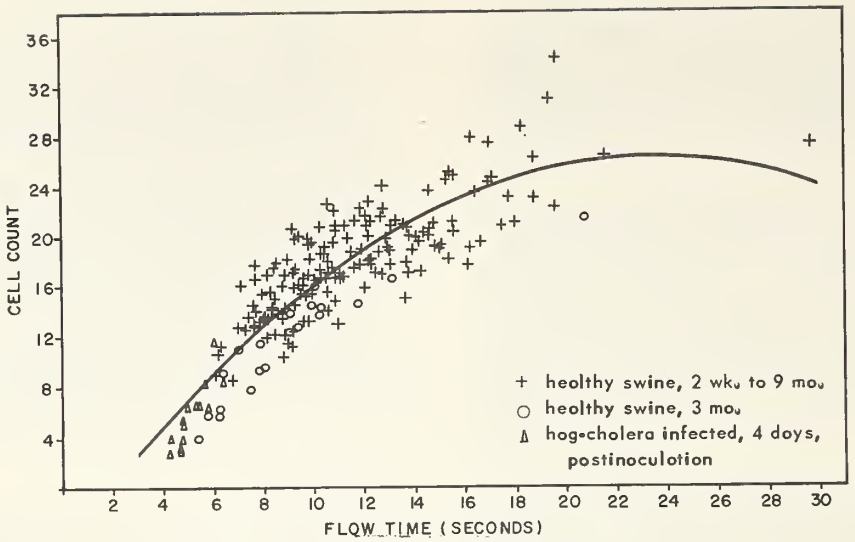

Figure 1.–Flow time–leukocyte number relationsip.

we are 95 percent confident that the true mean lies in this interval.)

Reagent.–No significant differences were observed when the commercial reagent was used in place of the concentrate which is diluted just before use. The frozen reagent became a yellow-green; but after thawing, returned to its original purple. The analysis of variance using temperatures as treatments and animals as experimental blocks was performed by the randomized complete block design with subsampling. The F value for treatments with (2) and (27) degrees of freedom was significant.

Field Studies.–A total of 53 samples were evaluated during the field study. The DNA flow times vs. hemacytometer counts were evaluated only as acceptable or unacceptable on the following basis. If hemacytometer counts and DNA viscosity estimates produced leukocyte counts within the same range; for example, less than 10,000; 10,000 to 13,000; over 13,000, they were considered as acceptable. When the two procedures produced values that were in two different ranges, they were considered unacceptable. Of the comparative counts, 47 were considered acceptable. Two of the other 6 counts were less than 1,000 cells from being within the same range. If these two were acceptable, 92 percent of the counts would be within the same range. When the data from the two methods were subjected to statistical treatment using an analysis of variance, no significant difference was detected.

Discussion

Theoretically the basis of this reaction is the interaction of the cell nuclear deoxyribonucleic acid and the anionic detergent in the reagent. A basic assumption of this procedure is that all of the DNA in the blood is found within the leukocytes. An excess of detergent is present in the mixture; and, therefor, it seems logical to assume that the more blood leukocytes present, the higher the viscosity of the gel will be. The specificity of deoxyribonuclease (DNAase) in preventing the reaction or dissolving the reaction-product-gel seems to confirm that DNA is present. Although the DNA-Feulgen reaction is less specific, it also supports the latter assumption. Carper (2) states that during the reaction, the lysing action of the reagent ruptures the leukocyte and breaks the bonds holding the DNA in a tight coil and concurrently releases DNAase from the leukocytic lysosomes. The DNAase rapidly lyses the long strands of DNA, thus reducing the viscosity of the mixture. Carper (2) states that this reagent, containing sodium lauryl sulfate and 1 percent EDTA inhibit the action of deoxyribonuclease by binding the magnesium and manganese ions necessary for DNAase activity. Schalm and Murray (9) performed the test on blood from the same species containing 2.0 mg./ml. of EDTA without observing any of the benefits described by Carper. The lytic action of the lysosome enzyme, DNAase, is the reasoning given for the loss of the viscous gel when the funnel flow time is not determined soon after the reagent and blood are combined; although there is no concrete proof of these assumptions, the reaction does seem to follow this hypothesis within the limits described.

Animal-Flow Time Distribution.–Carper (2) utilized the linear regression procedure by the method of least squares to calculate two linear regression lines showing the relationship of capillary-flow time and total hemacytometer-leukocyte counts. He reported a correlation

coefficient for the line of 0.933. Schalm and Murray (9) determined the mean values for flow time and leukocyte numbers. The points were interconnected to form the mean curve line seen in figure 1. The data resulting from these latter studies when plotted as in figure 1 appear to best fit a quadratic curve. The relationship is definitely not linear as determined by several tests of significance comparing the linear vs. curvilinear relationships.

An examination of table 1 reveals the interspecies difference in DNA levels of the leukocytes from different species. Less leukocytes are required in blood from a pig than in blood from a dog to produce similar flow times. The differences in average DNA content per cell between dog, horse, cow, and pig leukocytes resulted in a funnel flow time of 9 seconds for each animal species as representative of the maximum normal leukocyte number per cubic millimeter.

Flow times of less than 7 seconds, with swine blood, indicate the possibility of leukopenia. In these cases the test should be repeated using two drops of blood. If the flow time is not increased the existence of a leukopenia is confirmed. An increase in flow time for double the amount of blood will produce an estimate of sample leukocyte number of one-half the conversion value.

The repeatability of estimating leukocyte numbers is best when the leukocyte count falls within the normal and low range. This is apparent from figure 1. When larger number of leukocytes are present, the viscosity of the fluid increases and the flow through the capillary funnel becomes less and less uniform. For this reason the use of an average flow time based on three tests of each blood sample is recommended.

A number of observations regarding the interpretation of test results have been described. The funnel flow time apparently overestimates the number of leukocytes in leukemic bovine blood. Vendrely (12) has reported that neoplastic lymphocytes preparing to undergo mitosis have a greater quantity of DNA than resting cells. White reticulocytes do not interfere with the test, the more immature nucleated erythrocytes that enter the blood during severe anemias and other periods of accelerated erythropoiesis do produce and increased flow time. Lipemic blood and an excess of EDTA anticoagulant in the blood sample also appear to adversely effect the accuracy of the test. The accuracy of total white blood cell counts is commonly overemphasized. Berkson, Magath, and Hurn (1) stated that for a leukocyte count of 7,000 per cubic millimeter obtained by counting 4 sq. mm. of the chamber of the count is determined significantly within ± 21 percent.

Funnels.–The funnel flow times obtained using commercial reagent only were substantially lower than those of Schalm and Murray (9). His values had a mean of 5.4 seconds with a standard deviation (s.d.) of 0.2 seconds and a 95-percent confidence interval of 5.0 to 5.8 seconds. Our corresponding values were 4.6 ± 0.48 s.d. with a 95-percent confidence range between 4.12 to 5.08 seconds. Carper (2) stated that only a funnel with a 5.6 second flow time allowed correct estimates of total white blood cell counts using the published calibration chart. He mentioned only three funnels in his evaluations. The results of this study and those of Schalm and Murray (9) do not agree with those of Carper and Mitten (2).

Reagent.–We were concerned that the temperature extremes to which the reagent might be exposed during use in the field might reduce or destroy the accuracy of the test. The results of this study indicate that the solution is stable over a wide temperature range such as might occur in a veterinary practitioner's car while making calls. While statistical analysis of the data from testing swine blood with the solution subjected to the three temperature extremes demonstrated a statistically significant difference, it was of no practical significance. This is caused by the treatment differences being less than the limits of accuracy of this procedure.

The color change that occurred in the reagent when solidly frozen at -51° C. for 16 hours was from the normal deep purple to a yellow green. This is apparently because of the metachromatic properties of the brom cresol purple. The color change is reversible and occurs as soon as thawing begins (11).

Conclusions

This procedure merits use where an estimation of total white blood cell numbers is needed. It should not be expected to attain the accuracy of electronic or manual counting methods.

The DNA content of mammalian somatic cells apparently is a constant factor in peripheral blood leukocytes. A few exceptions exist, for example, neoplasia.

It is recognized that the basis of this test is empirical; however, its use is still justified.

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Abstracts of Published Reports

Cabrey, E. A., Stewart, W. C., Young, S. H., and Richardson, G. C. Transmission of hog cholera by pregnant sows. Amer. Vet. Med. Assoc. Jour. 149: 23-30. 1966.

Hog cholera virus was detected in the tissues of baby pigs farrowed by sows which were exposed to infection during pregnancy. The fluorescent-antibody, tissue culture technique (FATCT) was used to isolate and identify the virus, and pig inoculation was employed for additional evidence in five of the six cases. The atypical nature of the hog cholera infections in these herds delayed recognition of the disease and emphasized the value of laboratory assistance in reaching a diagnosis.

The ability of the pregnant sow to harbor and transmit hog cholera virus was confirmed.

Carbrey, E. A., Stewart, W. C., and Young, S. H. The changing picture of hog cholera: Case studies. Amer. Vet. Med. Assoc. Jour. 149: 1720-1724. 1966.

The concept of hog cholera (HC) as an acute, fatal disease of swine should be expanded to include a disease characterized by chronic signs of illness, recovery following supportive treatment in older pigs, mortality only in young pigs, and baby pig losses with congenital defects.

Transmission of hog cholera virus by the pregnant sow at farrowing through the birth of baby pigs infected in utero with HC virus; infection of susceptible pigs by contact exposure to pigs recently vaccinated with modified live-virus vaccines; and illness and losses in young feeders and baby pigs associated with HC infections caused by virus strains of reduced virulence were observed.

Carbrey, E. A. When and how to stop using vaccines. Livestock Conservation, Inc., Annual Meeting, 1966 Proc., pp. 31 and 32. 1966.

Modified live virus vaccines should not be employed in a herd where the pregnant sows (either vaccinated or unvaccinated), or the baby pigs, are not strictly isolated from the rest of the herd.

Exposure of pregnant sows to vaccine virus can result in the continued presence of hog cholera virus on the farm. Therefore, the administration of modified live virus vaccine to breeding swine that are exposed during an outbreak is not a desirable alternative to a procedure of complete herd depopulation.

It is not desirable to vaccinate pigs of any age with modified live virus vaccine and subject the pigs to stress conditions, such as shipping, castration, and worming.

Back passage studies would be helpful to determine which live virus vaccines, if any, might revert to virulence following four to eight passages through susceptible pigs.

Project Reports

Muhm, R. L. An outbreak of duck virus enteritis (duck plague) in the United States.

A disease resembling duck plague (duck virus enteritis, DVE) was originally observed in the Netherlands in 1923, and again in 1930, when it was reported as fowl plague (1, 8). The condition was reported as a new virus disease in 1942 (2). Since 1949, Jansen and others have done considerable research on this disease (4, 5, 6). Many members of the order Anseriformes (ducks, geese, and swans) have been found susceptible (3). In addition to the Netherlands, the disease has been reported in Belgium, China, France, and India.[1] In these outbreaks, the disease occurred principally in adult birds.

DVE was first reported in the United States on Long Island in January 1967.[1] The disease was diagnosed at the Cornell University Duck Research Laboratory (DRL), Eastport, N.Y. The outbreak occurred on four adjacent premises, making up one duck farm. The disease was suspected because of the distinctive lesions observed when the birds were necropsied. Virus isolations were made at the DRL and the Plum Island Animal Disease Laboratory (PIL). The disease was confirmed by serum neutralization (SN) tests at the PIL.[2] The only ducks on the affected farm were breeders, just coming into production. In February, after a period of observation with no outbreaks on other farms, the affected flocks were destroyed; and the birds and their byproducts buried.[3]

Toward the end of March the disease was reported in necropsy accessions from two other farms. In these outbreaks, the disease occurred in birds as young as 26 days.[1] Random blood samples were collected on 11 of the 40 duck farms on the island. Serums from four of these 11 flocks were positive for antibody on DVE on a SN test.

The Long Island duck industry is unique in many respects, and the epidemiologic aspects of a disease outbreak are very interesting. Approximately 8 million market ducklings are produced annually on 40 farms. Most of these are near or adjacent to other duck farms. They are located along inlets or coves which are tidal and contain brackish or salt water. The ducks, all White Pekins, can reach a weight of 7 lb. at 7 weeks of age. The pens are not large and contain concrete troughs of fresh, flowing water. The ducks have no contact with, and, in fact, cannot tolerate salt water. Most of the farms are integrated. Many have their own hatchery, and some have their own processing plant. There is a constant interchange of eggs and ducklings between hatcheries and between farms. A flock ready for market might be processed at more than one plant. While dressed duckling for human consumption is the primary purpose of the industry, there are numerous byproducts. Ducklings and breeders are often sold to other growers. Eggs and ducklings are widely used for research purposes in the United States and in foreign countries. The feet are shipped to Hong Kong and other Chinese communities where they are considered a delicacy. Raw and rendered offal may be shipped anywhere for use in animal foods. There is a render-plant that processes little except duck byproducts. A plant washes, dries, fluffs, and bags feathers to be used by the bedding industry. A concern collects, cleans, bales, and resells burlap feed bags. Wild birds, including gulls, ducks, and swans often mingle with the tame flocks and pass freely from one farm to another.

A program for the control of DVE was undertaken by the DRL and the Animal Health Division, Agricultural Research Service, U.S. Department of Agriculture. A part of this program was the examination of 1 day's mortality from each flock each week. In addition to the weekly submission, any unusual death loss was brought in for necropsy. Farms that had hatcheries (25) also submitted 150 eggs from their 7 to 10 day candling for virus examination. During a 60-day period (May 8 to July 7), 5,150 market ducklings and 290 breeders were necropsied. Each bird was closely examined for gross lesions of DVE. In suspicious cases, tissue samples were taken for pathologic and virus examination. DVE was sometimes complicated by bacterial and viral infections, and other conditions resembling the disease were frequently observed. The two conditions most commonly seen at the necropsy table were duck virus hepatitis and infectious serositis.

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[2] Dardiri, A. H., Hess, W. R., Breese, S. S., Jr., and Seibold, H. R. Characterization of duck plague virus from a duck disease outbreak in the United States. Presented at the 39th Annual Meeting of the Northeastern Conference on Avian Diseases, State University of New York, Stony Brook, N.Y. 1967.

[3] Newcomb, S., and Urban, W. Personal communication. 1967.

The incubation period of DVE is 3 to 7 days. The clinical signs are inappetence, thirst, and listlessness. There may be an ocular discharge and a watery diarrhea. The birds may be weak and unable to stand (5).[4]

Gross lesions of DVE at necropsy may include multiple petechiation and ecchymoses of the myocardium, the mucosal surface of the esophagus and cloaca, and the serosal surface of the digestive tract. The cloaca may be extremely congested, with bile-stained areas of necrosis at the posterior margin. Liver lesions are quite distinctive. The organ may be copper-colored with small red and white foci of hemorrhage and necrosis throughout. There may be an annular band of hemorrhage at the junction of the esophagus and proventriculus. Hemorrhages of the ovary often occur and free blood is sometimes observed in the abdominal cavity. Necrotic plaques may occur on the mucosal surface of the esophagus (5).[5]

The histopathologic lesions of DVE are most prominent in the liver and in the epithelium of the esophagus and cloaca. Microscopic examination of the liver may reveal numerous areas of necrosis and hemorrhage (figs. 1 and 2). Necrosis of the epithelial cells of the esophagus (fig. 3) and cloaca may be observed. The cytoplasm of the affected cells may contain viral inclusions.[5]

Duck virus hepatitis is a disease of ducklings characterized by hepatic hemorrhage and necrosis (fig. 4) and kidney degeneration. The histopathology of the liver lesions closely resembles that of DVE. Cytoplasmic viral inclusion bodies may be observed in the hepatic reticuloendothelial cells (7). The disease affects only young birds, differing from DVE in this respct. It cannot be reproduced experimentally after about 3 weeks of age.

Infectious serositis is characterized by fibrinous deposits and adhesions in the air sacs, paricardium, and peritoneal cavity. In most cases, *Pasteurella anatipestifer*

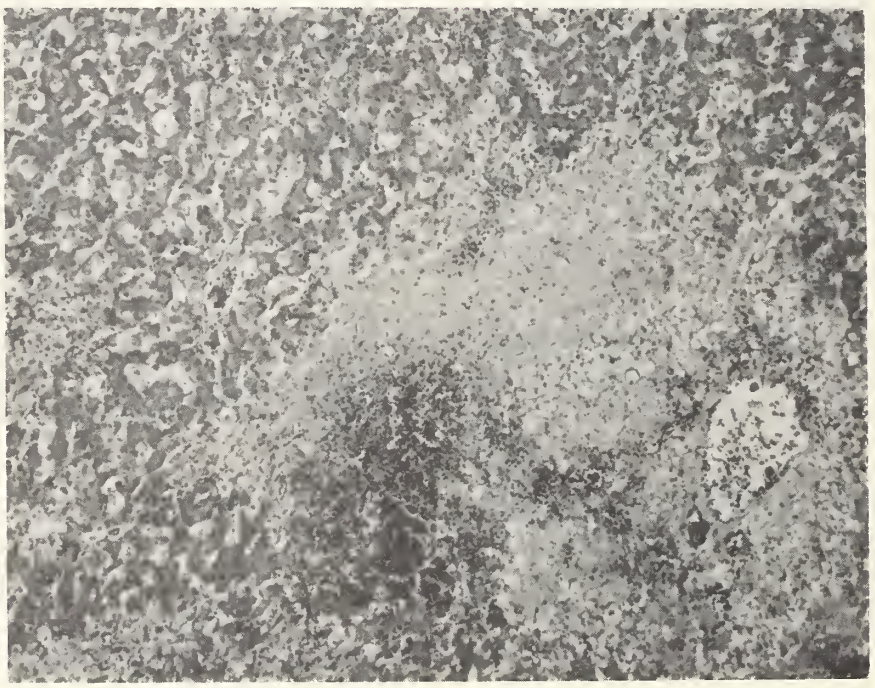

Figure 1.—Necrosis and hemorrhage in the liver of a duck that died of duck virus enteritis. (The magnification of these photographs was not calculated because a camera with a bellows was used.)

[4] See footnote 1.
[5] See footnote 1 and 2.

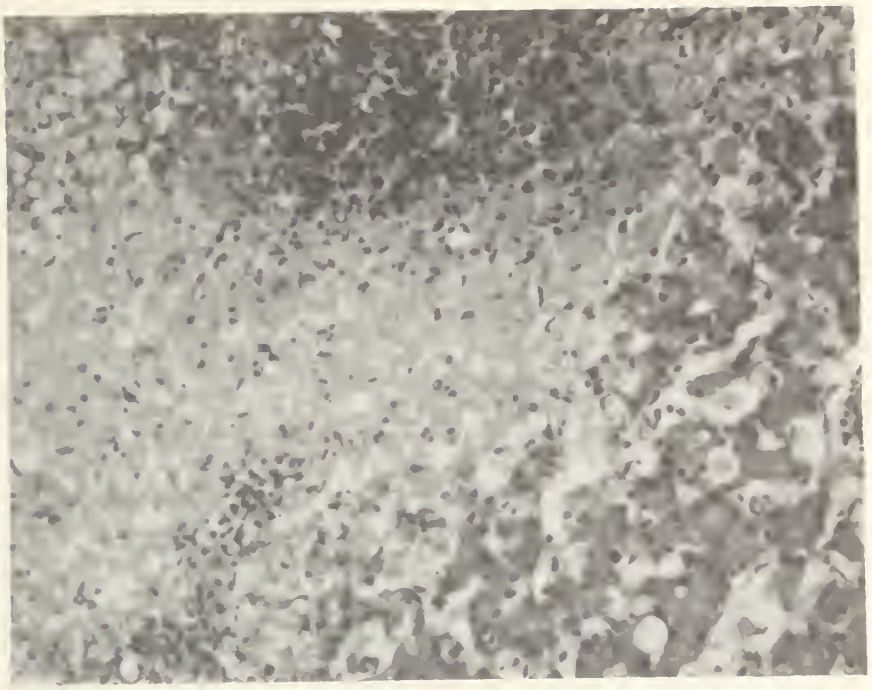

Figure 2.–Higher power of Figure 1. Necrosis, hemorrhage and fatty metamorphasis.

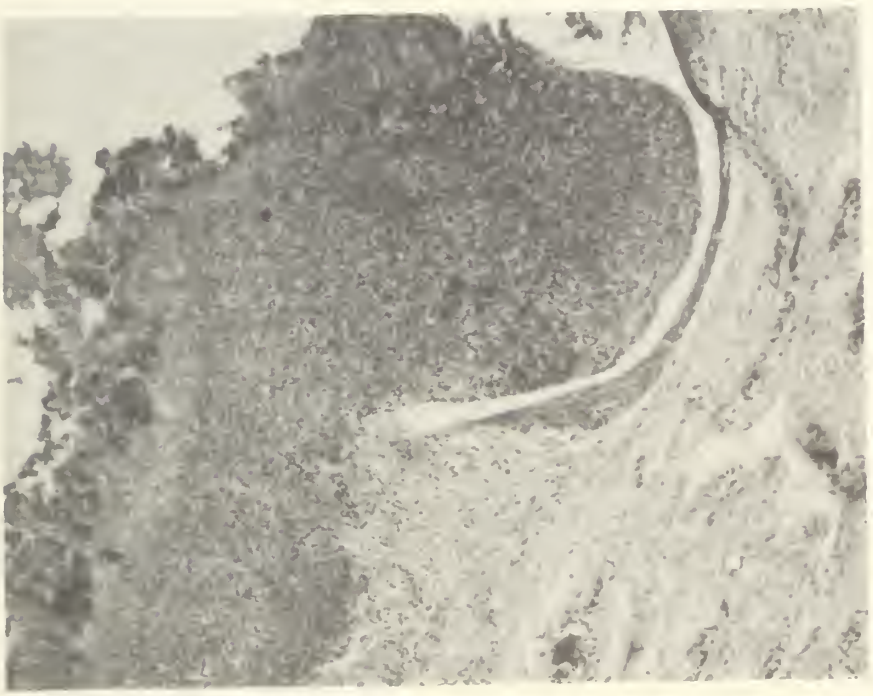

Figure 3.–Epithelial necrosis and exudate in esophagus of a duck that died of DVE.

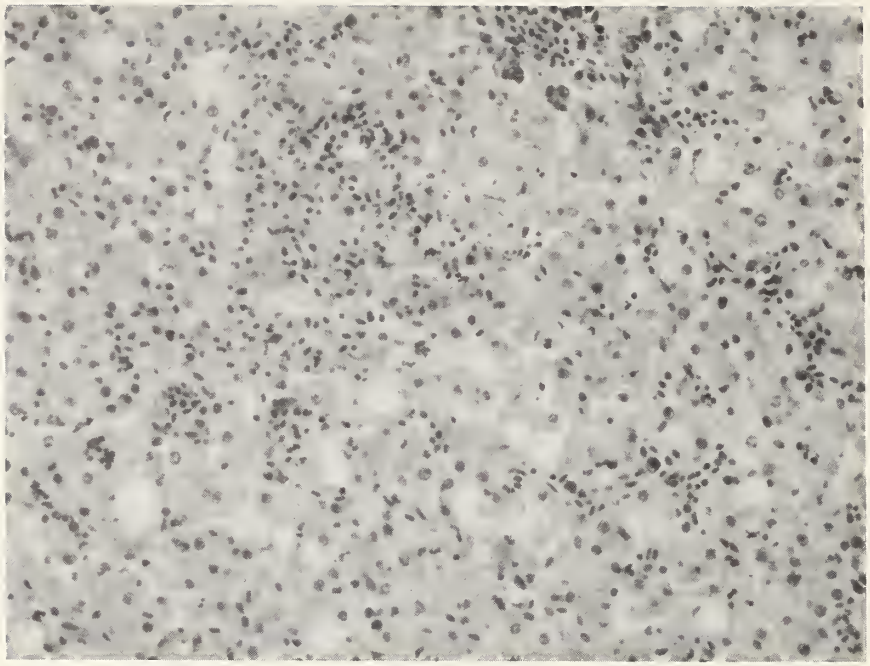

Figure 4.—Necrosis and congestion in the liver of a duck that died of DVE. (Note resemblance to fig. 2.)

is isolated on culture, occasionally *Escherichia coli,* and, in some instances, both organisms.

Other conditions, such as aspergillosis, paratyphoid, omphalitis, peritonitis, ascites, prolapse, necrotic enteritis,and injury were occasionally observed.

No DVE in commercial ducks was observed at necropsy for the 6 weeks following May 15. However, the disease was reported in Muscovy ducks from two ornamental flocks. Since that time, further outbreaks occurred in commercial flocks.

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Brown, C. L. and Jenney, E. W. Fluorescent antibody techniques for vesicular stomatitis virus.

Introduction

In developing a fluorescent antibody (FA) test that would complement present methods for the diagnosis of vesicular stomatitis (VS), three techniques were studied–direct, indirect, and complement stain.

The direct stain technique is based on the direct application of fluorescein-labeled antibody prepared against the disease agent to the infected cells. Hopkins and Jenney[1] described a direct FA stain for VS that differed from this report in the method of preparing conjugates.

The method of preparing coverslips for FA staining and the fluorescence observed in a positive test are described later in this report.

The indirect staining technique, as the name implies, is an indirect approach used to test serum samples. Unlabeled serum is first allowed to react with antigen. Fluorescein-labeled type specific antiserum produced in rabbits directed against homologous serum is then applied. A positive FA test depends on the unlabeled serum containing antibodies.

The complement (C') stain technique is similar to the conventional complement-fixation (CF) test, differing only in the mode of detecting fixation of C'. Whereas sensitized red blood cells detect fixation of complement in conventional tests, fluorescent antibody is utilized in the C' stain technique.

Public Health Publication No. 729[2] provides a detailed description of the FA techniques utilized in this work.

Materials and Methods

Antisera.–Bovine, equine, human, and swine serums from field cases and experimental inoculations were studied by the complement and indirect FA techniques.

Conjugates.–Serums from guinea pigs that were hyperimmunized with VS virus[3] were conjugated for the direct stain.

Serums from rabbits that were hyperimmunized with bovine and equine serums were conjugated for the indirect stain.[4]

Antigen.–Laboratory strains of New Jersey and Indiana VS virus were propagated in primary embryonic bovine and swine kidney tissue cultures grown on coverslips. A few positive VS specimens from field accessions were also inoculated into tissue culture.

Fractionation.–Ammonium sulfate precipitation of crude globulin was used for fractionating guinea pig and rabbit antiserums. Sulfate ions were removed from the protein solution by dialysis against 0.85 percent saline.

Serum neutralization (SN) and precipitation tests were conducted on some of the antiserums to check antibody titer before conjugation.

Protein Determination.–Protein determinations were made by the biuret colorimeter method using a spectrophotometer set of 540 mμ.

Conjugation (Combining fluorescein with antibody protein).–Protein fractions were adjusted with 0.85-percent saline to contain 1-percent protein. Fluorescein isothiocyanate (FITC) was added in a ratio of 1 mg. dye to 20 mg. of protein. The dye and protein were mixed overnight at 5° C.

Conjugates prepared from rabbit antiserums were tested by the precipitin test to measure antibody titer. The fluorescein-labeled protein was passed through a Sephadex (GS-25 course) chromatographic column to absorb and separte unreacted fluorescein.

Labeled antibody was absorbed with rabbit liver tissue powder to remove conjugated nonantibody protein. After precentrifugation in a PR-2 International refrigerated centrifuge at 1,250 g to sediment coarse particles of liver powder, the conjugate was recentrifuged in a Spinco Model L for 1 hr. at 30,000 r.p.m. (57,000 g) to remove fine particles.

Fluorescein to Protein (F:P) Ratio

Numerical estimates based on spectrophotometer measurements were used as guides to determine the fluorescein content of the conjugates. Concentration of fluorescein was determined by measuring percent transmission of conjugates on a spectrophotometer set at 480 mμ.

[1] Hopkins, S. R., and Janney, G. C. The Fluorescent Antibody Technique Applied to Vesicular Stomatitis Virus Using Serums Fractionated with Fthodin. Amer. Jour. Vet. Res. 23: 603-607.

[2] Cherry, W. B., Goldman, M., Carski, T. R., with collaboration of Moody, M. D. Fluorescent antibody techniques in the diagnosis of communicable diseases. U.S. Public Health Service Publication No. 729, 71 pp. 1961.

[3] Jenney, E. W., Mott, L. O., and Traub, E. Seriological studies with the virus of vesicular stomatitis, I typing of vesicular stomatitis viruses by complement fixation. Amer. Jour. Vet. Res. 19: 993-998. 1958.

[4] Proom, H. The preparation of precipitating sera for the identification of animal species. Jour. Path. Bact. 55: 419-426. 1943.

Fluorescent Antibody Staining Technique

Tissue culture coverslips were inoculated with 10^{-5} and 10^{-6} dilutions of VS virus and incubated 18 hours at 37° C. The coverslips were removed, washed in phosphate buffered saline (PBS), fixed in acetone for 20 minutes, washed twice in PBS, and allowed to dry. Coverslips were stained by the three FA techniques: direct, indirect, and C'. Rhodamine bovine albumin (RBA) at 20-percent concentration was used as a counterstain with some conjugates. This dye produced an orange background which contrasted with the yellow-green fluorescence of fluorescein.

Fluorescent Microscopy.—A Leitz Ortholux microscope fitted with a dry dark-field condensor was used for viewing. The light source was an Osram HBO 200 mercury arc lamp. A heat barrier filter, BG-38, was placed between the lamp and collector lens. A BG-12 (3 mm.) primary filter was used in conjunction with an OG-1 secondary filter. Employing this light source and filter system, the cytoplasm of the virus-infected tissue culture cells fluoresced yellow green against a dark background. When RBA was applied as a counterstain, the nuclei of infected cells, as well as normal cells, fluoresced a dark orange.

Direct Stain.—Conjugates prepared from New Jersey (N.J.) VS and Indiana VS hyperimmunized guinea pig serums were tested for antibody titer, specificity, and staining quality. Twenty-three tests were carried out by using coverslips infected with laboratory strains of VS virus. Bovine epithelium from four field cases and tissue from an experimentally infected hog were examined.

A direct stain was made on coverslip cultures infected with a virus isolated from an insect pool of *Hippelates pusio* (eye gnats) collected on a VS infected premise in Colorado.

Indirect Stain.—Forty-four tests were conducted on serums from cattle and guinea pigs that were artificially infected with VS virus and three tests performed on known positive horse serums. Thirteen samples comprising 17 cattle and one horse serum from field outbreaks of VS were tested and compared with conventional methods.

Complement Stain.—Box titrations were carried out with guinea pig C', RBA, known positive serums, and conjugates to test for optimum concentration of each reagent to be used in the FA test.

Forty-five FA examinations were conducted on artificially infected bovine, guinea pig, and hog serums by the C' stain technique and the results analyzed for sensitivity, specificity and staining quality. Seventy-one field serums comprising 39 bovine, 29 equine, and 1 each from human, sheep, and goat were also tested.

Results

Protein Determination.—Percentage protein of rabbit serum fractions ranged from 2.3 to 3.91 percent. Guinea pig antiserums ranged from 2.65 to 4.25 percent. The conjugate prepared from guinea pig serum containing 2.65 percent protein produced very dim fluorescence and was discarded.

Conjugation.—Precipitin titers of rabbit antiserums, used in the C' and indirect FA techniques, ranged from positive to incomplete at 1:16,000 dilution before conjugation. After conjugation, these serums had a fourfold to eightfold drop in titer.

Characterization of Conjugates.—Fluorescein to protein ratios ranged from 0.4 to 1.5×10^{-3} using dye from Nutritional Biochemical Corporation.[5] Better ratios ranging from 7.3 to 16.5×10^{-3} were obtained with dye from Baltimore Biological Laboratories.[6]

Direct Stain.—Conjugates prepared for direct staining had good staining quality when diluted out as far as 1:16 with or without counterstain. Bright-green fluorescence was observed in 23 tests with laboratory strains of VS virus and no evidence of cross or nonspecific reactions was detected.

Bovine tongue tissues from four outbreaks in Colorado and Texas and hog tissue harvested during a training school were identified in tissue culture by FA as New Jersey type VS virus. During the study of the epidemiology of VS virus, a direct FA stain identified a virus isolate from a pool of eye gnats as N.J. type VS virus. Subsequent tests by conventional methods confirmed the isolate as N.J. VS virus.

Indirect Stain.—Conjugates prepared from rabbit anti-bovine and antiequine serums produced bright-green fluorescence not unlike that observed in the direct stain. Best contrast between VS-virus infected cells and normal cells was observed by using RBA as a counterstain.

Of the 47 tests performed with known VS positive serums, 29 were positive and specific, three had a cross-type reactions, and 15 were negative.

Four of 13 field serums were positive by FA, three of 13 were suspicious by conventional CF tests, and 10 of 11 serums were positive by the SN test. Of the 4 serums positive by FA, three were suspicious by CF.

CF Stain.—Bright and easily definable stains were produced using guinea pig C' and RBA counterstain at 20-percent concentration with conjugates diluted from 1:8 to 1:16. Optimum concentration for testing antiserums was 20 percent, but further dilution was occasionally necessary to identify the virus type.

[5] Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

[6] Baltimore Biological Laboratories, Baltimore, Md. 21204.

From staining results of 45 tests on known positive bovine, guinea pig, and hog serums, it was shown that 27 tests were positive and type specific, four were cross type, and 14 failed to stain.

After testing 71 field serums by FA and comparing the findings with conventional CF tests, complete agreement was found.

Discussion

Conjugates prepared for the direct stain were interesting in that even though guinea pig serums crossed with heterologous antigen in the CF test, these same serums were specific in FA tests.

A continued study is planned for the diagnosis of VS virus in tissue culture by the direct stain technique.

It was hoped that conjugates prepared for the indirect FA test would prove useful in detecting antibody in convalescent serums. Our results from field sample serums indicate that antibodies in many SN positive serums were not detected by this FA technique. Those serums that were positive by CF were also positive by the indirect FA test.

The FA positive results reported by Seibold[7] during early convalescence seem to parallel our findings of positive FA during early convalescence (CF positive period) and negative FA results during later convalescence while the SN titer remained high.

The C' stain technique and the indirect stain have not proven useful in detecting antibody in late convalescent serums.

In a few tests the CF-stain technique has had cross-type reactions at low serum dilutions. The FA test is similar to the conventional CF test in this respect. However, when the serums are diluted out, the virus type can be differentiated.

Judging from results of the C' stain as applied to serial bleedings from a VS-infected hog, the FA test may prove useful with cross type and anticomplementary serums.

Jenney, E. W. and Brown, C. L. Studies on the epidemiology of vesicular stomatitis virus.

Introduction

In the United States vesicular stomatitis (VS) has been known to occur since 1916 and probably occurred before then (5). Annually a large sum of money is spent on its diagnosis to differentiate it from foot-and-mouth disease. VS has been studied by artificial inoculation, yet very little is known of its epidemiology. Some workers believe it is an arbovirus, some a plant virus, and some try to isolate it from fungi. Known arthropod isolations include four from *Phlebotomus* sandflies (3), two from mites (7), and two from mosquitoes (2, 8). Studies have shown that VS virus multiplies well and is transmitted by artificially inoculated arthropods (1).[1] This project was undertaken in an effort to contribute to the epidemiology of VS virus in our livestock. It includes serological studies of infected herds, viremia studies, and virus ioslation efforts from arthropods.

Materials and Methods

Serum Samples.—A large outbreak of New Jersey type VS occurred in Georgia and Alabama during 1963. Partly on the basis of previous history of VS, 12 Georgia herds were designated as VS study herds. Serums were collected from these herds during June and August 1963.

Thirteen hundred and one serums were tested from 1963 bleedings made 60 days following the New Jersey VS vaccination in Georgia (9).

The serums were screened by complement fixation (CF) in a 1:5 dilution against normal tissue, New Jersey VS, and Indiana VS antigens, and by serum neutralization (SN) employing 8-day embryonated eggs against New Jersey VS virus. Methods used were described by Jenney and Mott for the CF test (6) and Geleta for the SN test (4).

In the absence of clinical VS in the area, these herds were not rebled until the summer of 1966.

Veremia Studies.—To determine if a viremia existed during acute VS infection, a total of 96 blood samples were taken twice daily from seven cattle and three horses during the first 5 days of postinoculation. These blood samples were ground by mortar and pestle and inoculated into primary swine kidney monolayer tissue cultures. One ml. of each sample was inoculated into a 4 oz. prescription bottle, and at 3-day intervals subinoculations were made into primary swine kidney culture tubes for a total of three passages.

Arthropods.—During 1963, hematophagous insects were collected and identified. During 1964 and 1965, small insect collections were received from Georgia and Colorado.

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[1] Grayson, M. Arthropods as potential vectors of the virus of vesicular stomatitis. Presented at the First Veterinary Congress of Panama and Central America, August 12-15, 1964. (Translated by Emergency Diseases Staff, ADE, ARS.)

During 1964, to develop proficiency in arbovirus work, 277 mosquitoes were collected and identified, and virus isolations attempted.

During 1965, in the absence of large insect collections in the field, over 1,000 mosquitoes were collected at the NADL Animal Production Unit adjacent to Skunk River in Ames. These were processed to isolate arboviruses.

Virus isolation methods generally followed those described by Sudia and Chamberlain (10) and by work (11). Insect pools were ground with phosphate buffered saline containing 10-percent rabbit serum as a virus protectant, streptomycin, and penicillin. Each pool was inoculated intracerebrally into a litter of 1-day old suckling mice. If the death pattern of inoculated mice was suspicious for the presence of virus, mouse brains were aspirated and additional passages were made. If the death pattern continued on passage, crude or sucrose acetone extracted antigens were prepared from mouse brains. These were tested by complement fixation, hemagglutination, and hemagglutination-inhibition with goose red blood cells, and by neutralization tests to identify the isolate. Reisolation by virus from the original insect pool was attempted with each isolate.

In several cases, hyperimmune mouse serum was produced against the isolated virus using 1-month old mice. Lacking the necessary reference reagents for all of the arboviruses, six isolates were sent to the Arbovirus Reference Laboratory located at the National Communicable Disease Center at Atlanta, Ga.

Results

Serology on VS Study Herds.– Of 539 "preseason" serum samples, one was positive to New Jersey VS (table 1); four serums that were suspicious to Indiana type VS by the CF test were negative to Indiana VS by the SN test. One additional serum was suspicious to the CF test for New Jersey VS and two serums gave positive CF reactions with normal membrane antigen at the time of vaccination with egg origin vaccine.

Among 499 postseason samples, 10 were reactors and 11 suspects to New Jersey VS virus. Clinical VS had been reported from two of the farms with reactors,but no clinical disease was observed on three of the farms with CF positive cattle. Four serums gave cross type or nonspecific reactions; three reacted to normal egg antigen. Of these serums, 27 were anticomplementary.

Serology on VS Vaccinated Herds with Clinical VS During 1963.–In a group of 741 serums samples of which 541 were collected from vaccinated cattle (table 2), five positive and 13 suspicious serums were detected by CF. Five serums had positive reactions against normal egg antigen.

Serology on VS Vaccinated Herds without Clinical VS.–With 560 serums, 431 of which were vaccinated, there were one reactor, four suspects, two cross type reactions, three reactions to normal membrane antigen, and two anticomplementary serums.

CF reactions to normal membrane antigen increased from 0.35 percent (2/539) in prevaccination bleeding of study herds to 0.9 percent (11/1208) in the postvaccination bleeding. About two-thirds of the animals were vaccinated in these herds. Three of the 11 reactors to normal membrane antigen were nonvaccinated.

Viremia Studies.–No virus was isolated from the 96 blood samples collected from cattle and horses following intradermal inoculation with New Jersey VS virus.

Virus Isolation Efforts from Arthropods.–A summary of the virus isolation attempts from arthropods is presented in table 3.

Three isolations of nonhemagglutinating viruses were made from pools of *Culex salinarius* collected on neighboring farms during the first 2 weeks of August 1963. These three isolates were identified as Flanders virus by the Arbovirus Reference Laboratory.

Western encephalitis (WE) virus was isolated from one pool of mosquitoes collected by a light trap located in the milking shed at Florence, Colo., during the 1964 VS outbreak. The Colorado entomologists making this collection identified the pool only to *Culicinae.* Other insect pools collected in Colorado during 1964 were negative.

Seventeen pools of tabanids and other diptera collected in Georgia during 1964 were also negative.

Trivittatus virus, a member of the California encephalitis group, was isolated from a pool of *Aedes trivittatus* collected at Ames, Iowa, during 1964. This was one of the first isolations of trivittatus virus from east of the northern Rocky Mountain States and North Dakota. The isolate was identified by the Arbovirus Reference Laboratory.

No isolations were made from the insect pools collected in Colorado during 1965.

One WE and two nonhemagglutinating viruses were isolated from the 1,000 mosquitoes trapped at the Animal Production Unit of the National Animal Disease Laboratory during summer of 1965. The WE virus was isolated from a pool of *Culex tarsalis* collected during September, while clinical encephalitis was known to be occurring in equines about 12 miles down river. Isolations of nonhemagglutinating viruses were made from a pool of *Culiseta inornata* and from a mixed pool of *Culex salinarius, C. restuans,* and *C. pipiens.* These isolates were both identified as Flanders virus by the Arbovirus Laboratory at the National Communicable

TABLE 1.–Summary of complement-fixation tests on serums from study herds, Gerogia, 1963

Owner	Prescason prevaccination bleeding, June 1963						Postscason bleeding, July-August 1963							
	Tested	Positive	Suspect	Negative	Cross type	Normal membr. Antigen	Tested	Positive	Suspect	Negative	Nonspecific or cross type	Normal membr. Antigen	Vaccinated	VS history 1963
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
SHS	38	0	[1] 3	35	0	0	47	1	1	[3] 38	0	1	18	?
GH	33	0	0	32	0	1	32	0	0	30	0	0	17	-
HG	54	0	0	54	0	0	51	1	1	44	0	0	23	[2] -
RJ	30	0	0	29	0	1	40	2	0	38	0	0	20	+
LS	87	1	0	85	1	0	99	3	6	75	2	2	40	+
GH	38	0	0	38	0	0	16	1	0	7	1	0	8	[2] -
RA	43	0	0	43	0	0	42	0	0	38	0	0	20	+
BEE	53	0	[1] 1	52	0	0	52	0	0	50	1	0	26	-
LS	27	0	0	27	0	0	25	0	0	25	0	0	14	-
JAMW	62	0	1	61	0	0	63	2	3	58	0	0	25	[2] -
SES	51	0	0	51	0	0	32	0	0	30	0	0	25	?
LT	23	0	0	23	0	0	–	–	–	–	–	–	–	–
TOTAL	539	1	5	530	1	2	499	10	11	433	4	3	236	

[1] Suspicious to Indiana-type VSV - negative to Indiana VS on neutralization tests.
[2] No clinical VS during 1963 specifically mentioned; VS present in herd adjacent to HG.
[3] Discrepancy in totals are 27 anticomplementary serums from apparent mishandling of postseason bleeding.

TABLE 2–Complement-fixation serology findings on vaccinated herds

Herds with clinical VS during 1963							Herds without clinical VS during 1963						
Code	Tested	Vaccinated	Positive	Suspect	Cross type	Normal membr. antigen	Code	Tested	Vaccinated	Positive	Suspect	Cross type	Normal membr. antigen
	Number	*Number*	*Number*	*Number*	*Number*	*Number*		*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
B	41	25	0	0	0	1	A	23	15	0	2	0	0
RB	56	55	2	4	0	0	RC	16	16	0	0	0	0
C	67	60	1	1	0	2	CA	26	25	0	0	0	1
FA	61	61	1	1	0	0	CB	49	30	0	0	0	0
FH	67	62	1	0	0	0	CD	44	30	0	0	0	1
RA	65	50	0	0	0	0	CE	39	30	0	0	0	0
RE	30	15	0	0	0	0	CF	2	2	0	0	0	0
G	90	78	0	0	0	0	D	45	40	1	0	0	0
P	50	30	0	0	0	0	E	104	77	0	0	[1] 0	0
L	26	26	0	0	0	1	FB	84	62	0	0	[2] 2	0
T	88	49	0	3	0	1	FC	14	14	0	0	0	0
U	57	30	0	4	0	0	FG	12	12	0	0	0	0
X	43	0	0	0	0	0	RF	10	10	0	0	0	0
							I	23	0	0	0	0	0
							H	29	29	0	0	0	1
							M	40	40	0	2	0	0
TOTAL	741	541	5	13	0	5		560	431	1	4	2	3

[1] One serum each anticomplementary for E, FB, and FC.
[2] Three plus reactions to both N.J. and Indiana antigen; negative to normal.

TABLE 3.–Arbovirus isolation efforts in suckling mice from 1963 to 1965

Year of collection	State	Pools	Identification[1]	Findings
		Number		
1963	Georgia	85	Various mosquitoes and tabanids	Negative.
1963	do	3	Culex salinarius	Flanders virus.
1964	do	17	Tabanids and other diptera	Negative.
1964	do	39	Various diptera and 1 reduviidae	Do.
1964	do	1	Mosquitoes	Western encephalitis.
1964	Iowa	7	do	Negative.
1964	do	1	Aedes trivittatus	Trivittatus virus .
1965	Colorado	15	Mosquitoes, diptera, few miscellaneous	Negative .
1965	Iowa	34	Mosquitoes	Do.
1965	do	1	Culex tarsalis	Western encephalitis.
1965	do	1	Culex pipiens, C. restuans	Flanders virus.
1965	do	1	Culiseta inornata	Do.

[1] Insects from the 1963 Georgia collection were identified by Jerry Sutter, the 1964 and 1965 Colorado collections by Colorado State entomologists, the 1964 Ames, Iowa, collection by Russell Wright, and the 1965 by E. W. Jenney.

Disease Center. Flanders virus is not known to infect mammals. Virus of the Flanders-Hart Park group has been isolated from birds.

Conclusions

Serology.–There appears to have been subclinical infection in the study herds as indicated by the presence of reactors and suspects in herds where clinical lesions were not reported; at least one case involved a farm adjacent to known infection. After comparing one reactor among the 431 vaccinated animals in the vaccine trials where VS was not observed (table 2) to five out of 181 positive cattle in three study herds where VS was diagnosed (table 1 herds RJ, LS, and RA postseason bleeding), or the five out of 318 positives where VS was not observed, it does not appear that positive CF reactions are produced by the vaccination. Vaccination of the cattle did not markedly interfere with serology of samples taken 2 months postvaccination; no significant increase of titer appeared either against the type specific antigen or to normal egg antigen.

Viremia Studies.–The failure to demonstrate a viremia in cattle or horses confirms work by other workers that, if viremia occurs, it is usually of a very low titer or of a very short duration. Blood samples should be collected more frequently to detect shorter periods of viremia if present. Knowledge of the presence or absence of viremia is of importance in studying the transmission of VS. If no viremia exists, the search for arthropod carriers and other means of transmitting the disease must be extended to include other than hematophagous insects, in particular as this relates to the livestock part of the virus cycle.

Arbovirus Studies.–Failure to isolate VS virus from mosquitoes in our studies decreases the likelihood that mosquitoes are a major vector in VS transmission. The isolation of the Cocal subtype of Indiana VS virus from *Culex* by Jonkers, of classical Indiana type VS virus from *Aedes* by Sudia, and the artificial propagation of New Jersey type VS virus in artificially infected mosquitoes must not, however, be overlooked. Viremia, although not demonstrated in cattle and horses, may be present in rodents, wildlife, swine, or poultry among which other transmission cycles may be at work enabling these animals to serve as reservoirs of infection. Certainly the preseence of epizootics of Cocal virus in rodents on Bush Bush Island as demonstrated by Jonkers is evidence of such a cycle. Insects such as *Hippelates*

eye gnats that feed on mucous should be considered in transmission among livestock. These are known to transmit mastitis and may be associated with udder lesions, mucous, saliva, and vesicular fluid of livestock infected with VS.

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Project Reports

Nelson, H. A. The use of gas chromatography in detecting lindane in sewage effluent.

Introduction

Gas-liquid chromatography appeared to be a rapid, convenient, and accurate means of detecting and measuring lindane, a chlorinated hydrocarbon insecticide.

Basic principles of gas-liquid chromatography are as follows:

1. Basic components necessary for gas-liquid chromatography are an inert carrier gas, column, detector, electronic components, and a recording.
2. The sample is injected into a chromatographic column, volatilized with heat, and transported through the column by the carrier gas. Retention time is the period of time any given component of the sample remains in the column. This depends on the volatility of each component and its solubility in the liquid portion of the column. A highly volatile compound that is slightly soluble has a short retention time; a less volatile and more soluble compound has a longer retention time. When operating conditions such as temperature, column length, column packing, and flow rate of carrier gas remain the same, the retention time for a specific compound is constant.
3. After passing through the column, the compound passes through the detecting device, and a signal is passed to the electronic equipment and recorded. The magnitude and duration of this signal are dependent on concentration of the compound.

An experiment was designed to see if lindane could be safely and effectively disposed of through an activated sludge sewage disposal system. The objective was to measure lindane in effluent and sludge after known amounts were added to a pilot plant.

Methods

Plant operation and sample collection.–A pilot plant of the complete mixed activated sludge type operated at approximately conventional activated sludge loading was located at a municipal sewage disposal plant. A part of the raw sewage was diverted into the pilot plant. Lindane was introduced into the plant at a rate calculated to correspond to the emptying of a dipping tank into a municipal sewage system over a 1- or 12-hour period.

One gallon samples of influent and effluent were collected before introduction of lindane and at intervals of 2, 4, 6, 12, 24, and 48 hours after introduction of lindane began.

Analysis.–Petroleum ether was first used to extract lindane from sewage samples and subsequently served as the lindane carrier when introduced into the gas chromatograph.

The operating conditions for lindane analysis were as follows:

1. Column: 6-foot coiled glass with inside diameter of ¼ inch. Packing used was Chromosorb W substrate[1] (60 to 80 mesh) and 10 percent Dow-Corning Silicone fluid.[2]
2. Detector: Packard Electron Capture[3] with 150 millicurie tritium source.
3. Temperature:

	Column	190° C.
	Inlet	225° C.
	Detector	195° C.
	Outlet	200° C.

4. Carrier gas: Nitrogen at 120 cc./min.

Results

Gas liquid chromatography was successfully used to accurately detect and quantitate lindane present in sewage effluent. The amount of lindane present was determined by comparing peak heights of samples to peak heights of known standards of lindane. Using this procedure, levels of lindane as low as 0.150 μg per gallon were measured.

Nelson, H. A. Lindane and toxaphene disposal.

Introduction

A safe and effective way to dispose of toxaphene and lindane dipping solutions has been a problem of major concern to the Animal Health Division. Many of the dipping tanks are located in heavily populated areas and disposal has been via municipal sewage disposal systems.

[1] Johns-Manville, New York, N.Y.
[2] Dow-Corning, Midland, Mich.
[3] Packard Instrument Company, Downers Grove, Ill.

A cooperative project was arranged between the Toxicology Unit of Diagnostic Services, National Animal Disease Laboratory, and the Iowa State University's Civil Engineering Department to determine if dumping the contents of a livestock dipping vat into the city sewerage system is a safe means of disposal.

The first stage of this project was conducted by Owen Sletten and R. H. Singer. The purpose was to establish the effects of toxaphene and lindane on an aerobic biological sewage treatment process. Results of this study indicated conventional aerobic sewage treatment processes would tolerate lindane levels as high as 600 mg. per liter and toxaphene concentration up to 3,000 mg. per liter without significant detriment to the plant. This study indicated only minor degradation of the presticides occurred and lindane would probably accumulate in the sludge (sediment) rather than effluent (liquid).[1]

Materials and Methods

In the next phase of the project a pilot plant was set up at the Ames, Iowa, municipal sewage disposal plant. Solutions of toxaphene and lindane were introduced to determine effects on plant operation and also to determine if the plant would remove or degrade the pesticide sufficiently to produce a safe effluent. The rate of adding the insecticide into the pilot plant was calculated to simulate the rate the insecticide would reach the sewage disposal plant following emptying a 9,000 gallon dipping vat.

The pilot plant was set up and operated by the Iowa State University Civil Engineering Department, and pesticide analytical work was conducted by Clinical Pathology and Toxicology, Diagnostic Pathology, Animal Health Division, National Animal Disease Laboratory.

Results

In the lindane trials amounts of lindane were added to simulate emptying a 9,000 gallon dip tank containing 600 mg. per liter into the sewage system of a city with a population of 3 million. The pesticide was added uniformly over 1- or 12-hour periods. The efficiency of the activated sludge process was not affected by addition of these amounts of lindane. Lindane was present in the effluent in all trials; however, the amounts were very small. The highest concentration found was 10 μg. per liter (0.01 parts per million). This occurred after a 1-hour emptying period. Accumulation of lindane in the sludge liquor was not so great as expected. Six percent of the lindane added was present in the mixed-sludge liquor 24 hours after starting the addition of lindane. An average of 80 percent of the added lindane was not accounted for in the effluent or sludge. It is presumed this portion was degraded biologically. Under the conditions of this expeimental work, it would appear that an activated sludge sewerage disposal system is a satisfactory means of disposing the lindane dip solutions.

In the toxaphene trials a significant amount of toxaphene passed into the effluent when the quantities of toxaphene introduced simulated emptying a 9,000 gallon dip tank containing 6,000 mg. per liter into the sewage systems of a city with a population of 30,000. Toxaphene was retained in the system for more than 3 weeks and during this time gradually passed into the effluent. The average level of toxaphene present in the effluent over a 2-week period was 1,100 μg. per liter. Toxaphene has been reported to be toxic to fish in levels as low as 5.1 μg. per liter.[2]

Limited degradation of toxaphene occurred, but most of it accumulated in the sludge and gradually passed into the effluent over a period of at least 3 weeks.

Summary

The results of this study indicate that an activated sludge sewage disposal plant is suitable for lindane disposal but not toxaphene.

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Project Reports

Cassidy, D. R., and McDaniel, H. A. Histopathologic studies of lung changes in scrapie and non-scrapie blood line sheep maintained in a scrapie environment.

Abstract

Histopathologic examination was conducted on lung tissue from 23 sheep that died during a study of scrapie. Lesions of scrapie were demonstrated in the brain of one animal. Pneumonic lesions were observed in 13 animals including the scrapie-infected animal.

Introduction

Several "slow" virus infections, including Visna, a viral encephalitis, and Maedi and Jaagsiekte, two progressive pneumonias of sheep, have been described by Sigurdsson (4, 5, 6). Lesions in both the lungs and central nervous system of Dutch sheep affected with Zwoegeziekte have been described by Ressange and coworkers (2). This author pointed out the similarity between the pneumonic lesions of Maedi and the encephalitic lesions of Visna, and the lesions observed in Dutch sheep affected with Zwoegerziekte. After the isolation of a virus from cases of Visna by Sigurdsson and coworkers (7), and from lungs of sheep affected with Maedi by Sigurdardottir and Thormar (3), a close relationsip between both viral agents in many of their properties was demonstrated (7, 8). The following study was initiated to determine if any association exists between scrapie and the chronic ovine pneumonias such as Jaagsiekte, Maedi, and Zwoegerziekte.

Materials and Methods

All sheep at the Mission Scrapie Study Project, Mission, Tex., were necropsied and tissues including the brain and sections of lung, were fixed in buffered 10-percent formalin. Lung tissues were impregnated with paraffin, section at 6 micra and stained with hematoxylin and eosin. Brain tissue was stained by the method of Cajal and the Azure-Phloxin method to demonstrate astrocytosis and the intracellular vacuoles typical of scrapie.

Results

The results of histopathologic examination of the brain and lung tissues are listed in table 1. Lung lesions were present in 13 of the animals including the scrapie-infected animal. *Erysipelothrix insidiosa* was isolated from lung tissue of the animal infected with scrapie and probably accounted for the purulent pneumonia.

In a number of cases the changes present in the lung were those of a chronic pneumonitis upon which changes of an acute bronchopneumonia were superimposed. Consequently, some of the more common changes observed in the chronic pneumonias were not clearly defined.

Discussion

When completed, this study should furnish information on a number of questions. Is Maedi present in the sheep population of this country? Does the causative agent of scrapie produce or predispose to a progressive pneumonia such a Jaazsiekte or Maedi? The findings of Ressang and others (2), indicate that the agent Zwoegerziekte may produce both encephalitic and pulmonary lesions. The relationship of Zwoegerziekte to Visna has not been reported. Although lung lesions have not been described in field cases of Visna, they have been experimentally produced (1). They resemble those seen in the early stages of Maedi. Scrapie and Visna have been reported to be caused by serologically distinct agents (5).

The findings described here are difficult to interpret at this stage because of (1) only a single scrapie-infected animal being available for comparison, (2) the tendency for chronic and partly healed bronchopneumonias to resemble "slow" pulmonary infections, such as Maedi and Zwoegerziekte in sheep, and (3) a lack of information on the biochemical changes occurring in lung tissue during the course of the "slow" infections. However, the results obtained indicate that chronic pneumonia from a variety of causes is not uncommon in sheep at the age when scrapie symptoms become apparent.

Future studies on normal lung tissue, those described here, and additional scrapie-infected tissues are indicated. Examination of Giemsa stained lung sections for the intracytoplasmic inclusions of Maedi will be carried out. Histochemical studies of lung lesions from both scrapie and non-scrapie affected sheep are in progress.

TABLE 1.–Results of histopathologic examinations

Accession No.	Brain lesions typical of scrapie	Lung lesions
25529	Negative	Tricuspid valvular disease.
25530	Positive	Chronic purulent pneumonia--E. insidiosa.
28090	Negative	No lung tissue available.
28298	do	Chronic focal suppurative pneumonia.
28299	No brain	Congestion.
28565	Negative	Chronic, focal suppurative pneumonia.
28566	No brain	No pathology specimen.
28567	Negative	No lung tissue in specimen.
29059	do	Chronic, suppurative pneumonia. Chronic, fibrinous pleuritis.
29060	do	No specific changes.
29500	do	Do.
29501	do	Severe suppurative and caseous bronchopneumonia, mild interstitial nephritis.
29502	do	Severe postmortem changes.
29799	do	Subacute pneumonitis.
29800	do	Severe postmortem changes.
29801	do	Suppurative, necrotizing pneumonia.
29802	do	Mild chronic pneumonitis.
29865	do	Severe chronic pneumonitis.
30008	do	Severe postmortem changes.
30009	do	Do.
30010	do	Chronic focal pneumonia.
30343	do	Mild chronic bronchopneumonia.
30352	do	Pulmonary edema, congestion.
30353	do	Mild chronic pneumonitis.
30354	do	Focal chronic pneumonitis.
30355	do	No lung lesions.

Differences in the relative amounts of reticular fibers and collogen in these tissues, as well as their chemical composition will be studied by these procedures.

The results may enable the development of diagnostic criteria for these and other diseases of the ovine lung.

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Project Reports

Sherman, K. C., and McDaniel, H. A. Nymphal stage of *Linguatula serrata* found in mesenteric lymph node of a bovine.

Introduction

Larval forms of the parasite *Linguatula serrata* are occasionally found encysted in the mesenteric lymph glands of cattle when slaughtered. The presence of this parasite in the lumph node stimulates a granulomatous reaction that is grossly indistinguishable from lesions of tuberculosis. A microscopic examination of the lesions is required to determine the etiology.

In the bovine tuberculosis eradication program, meat inspectors submit tuberculous suspicious lesions encountered during routine slaughter. Many specimens are examined for tuberculosis that are finally diagnosed as parasitic granulomas. Usually only fragments of a degenerated parasite are observed. Occasionally a fairly good cross section of the parasite is seen but rarely a good sagital section. In the specimen discussed in this report the parasite was ideally sectioned in a sagital plane.

The specimen was submitted from a slaughtering establishment in Montana. The lesions came from a bovine animal described as a young adult. According to the veterinary meat inspectors, the tissues had a greenish discoloration and contained caseous lesions with white and greenish granules in the exudate. The lesions were confined to the mesenteric lymph nodes.

Histopathology

The mesenteric lymph node was sectioned and stained with hematoxylin and eosin. The parasite was located in the center of the lesion surrounded by a purulent exudate containing many eosinophils (fig. 1 and 2). A definite gramulomatous epithelioid cell response surrounded the necrotic, purulent center of the lesion. A fibrous connective tissue capsule enclosed the entire lesion. Eosinophils are present, not only in the exudate but also infiltrated into the area of the granulomatous epithelioid cell response. The lymph node tissue near the lesion was densely infiltrated with

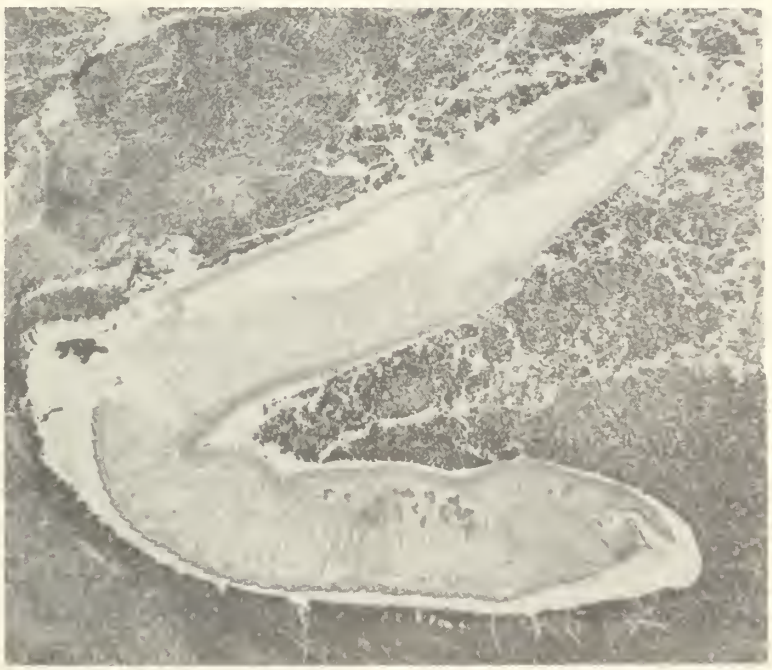

Figure 1.–Intact parasite *Linguatula serrata* surrounded by a purulent exudate composed primarily of degenerating neutrophils and eosinophils (approximately 40X).

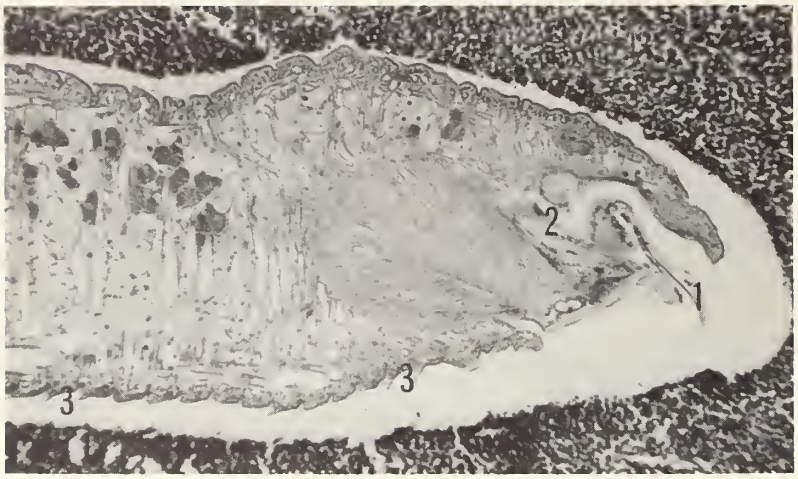

Figure 2.—Anterior end of the parasite: hooks (1), well-developed mouth parts (2), spiney cuticle (3) (magnification 100X).

eosinophils. These eosinophils gave the tissues a greenish discoloration as seen grossly.

Life Cycle

The adults generally occur in the nasal passages and frontal sinuses of dogs, but they are also found in other carnivorous animals, such as foxes, coyotes, and members of the feline family. Adults attach to the nasal mucosa and suck blood. Eggs are expelled in the feces of the host and in the nasal discharge. The eggs remain viable outside the host for long periods. Herbivorous animals, such as cows or rabbits, ingest contaminated grass. The eggs hatch in the intestines and the larvae migrate to the mesenteric lymph nodes where they become encysted. The larvae undergo several molts and develop into a nymph. The parasite is infectious at this stage. When ingested by another carnivorous animal, it migrates into the nasal passages and frontal sinuses where it becomes an adult.

Classification of Linguatula serrata

Linguatula serrata is classified in the order Pentastomida and infection with linguatulid larvae is sometimes called pentastomiasis. Pentastomida belongs to the class Arthropoda. At one time Pentastomida were classified in the order Arachnida and members were thought to be legless mites. This belief is no longer accepted and Pentastomida are now recognized as a separate order.[1] It is hard to recognize these parasites as arthropods as their life as parasites has greatly modified their appearance, but their larval forms still identifies them as arthropods.

[1] Chandler, A. C., and Read, C. P. Introduction to parasitology. Ed. 10. New York, London, John Wiley and Sons, Inc. pp. 562-564. 1962.

Abstracts of Published Reports

Greve, J. H. and Cassidy, D. R. Aberrant *Hypoderma bovis* infection in a cow. Jour. Amer. Vet. Med. Assoc. 150(6): 627. 1967.

An aberrant third instar *Hypoderma bovis* (de Greer, 1776) was found in a lymph node from a cow. Grossly, the resultant lymphadenitis resembled tuberculosis. The possibility is suggested that Hypoderma larvae may persist for several months anaerobically in bovine tissue.

Cassidy, D. R., Morehouse, L. G., and McDaniel, H. A. *Mycobacterium avium* infection in cattle, a review and series of cases. Amer. Jour. Vet. Res. (29(2): 405-410. 1968.

This paper summarizes the study of the largest known series of isolation of *Mycobacterium avium* from cattle in the United States. The study covers the findings in tissue specimens from 103 cattle from 24 States. The organism was isolated from normal and tuberculous tissues, from lesions of tuberculoid dermatitis, and of pulmonary ossification, as well as from those affected by actinobacillosis, neoplasms, and purulent lesions. The study shows that (1) the scattered source of the specimens suggest that the condition has a widespread geographic distribution in this country and (2) suggests that *M. avium* is capable of widely varying degrees of pathogenicity in cattle.

Project Reports

Ringham, Carole K. An analytical method for determining arsenic in biological material.

Introduction

An accurate and reliable analytical method was needed in this toxicilogy laboratory for determining arsenic in biological material. This is important in differentiating normal and abnormal levels found in animals due to therapy and other sources of exposure which are present when toxicity occurs.

The modified Gutziet method[1] was formerly utilized for detection and quantitation (which was subjective because of visual color comparison). The modified silver diethyldithiocarbamate (AgDDC) method[2] utilized the spectrophotometer for measurement of color development, was reproducible, and relatively simple to perform.

These two methods were compared by using standards containing known amounts of arsenic trioxide.

Materials and Methods

Reagents:

1. Silver diethyldithiocarbamate—0.5 percent in pyridine, analytical reagent (AR). Prepare 24 hours before using. Stable for 3 months if stored in cool, dark place:
2. Potassium iodide—15 percent weight/volume in distilled water. Prepare weekly.
3. Stannous chloride—20 percent weight/volume in concentrated HC1. Stable indefinitely.
4. Isopropyl alcohol.
5. Zinc granules—20 mesh.
6. Copper sulfate crystals.
7. Lead acetate glass wool—impregnate glass wool with 10 percent lead acetate and dry in oven.
8. Sulfuric acid (AR).
9. Nitric acid (AR).
10. Perchloric acid (AR).
11. Distilled or double-distilled water—preferably in a glass still.

Procedure:

1. Digest 10g. tissue or 10 ml. blood using 10 ml. nitric acid, 3 ml. sulfuric acid, and 0.3 ml. perchloric acid.
2. Dilute digestate to 100 ml. with distilled water.

Preparation of arsine generator:

1. Place approximately 5 g. of 20-mesh zinc granules into a 125 ml. Erlenmyer flask.
2. Add a few cupric sulfate crystals (1 to 2 mg.)
3. Moderately pack lead acetate glass wool into a glass tube and insert into a rubber cork that will fit the Erlenmyer flask.
4. A rubber tube with a serum pipette inserted in one end and the packed glass tube in the other will serve to transfer the arsine gas from the generator to the receiving tube.

Analysis:

1. Place a 2 g. (20 ml.) aliquot of digestate into a 150 ml. beaker.
2. Add 1.4 ml. concentrated H_2SO_4 and dilute to 0.5 ml. with 10 percent H_2SO_4. (Final concentration is 10 percent H_2SO_4.)
3. Add 1 ml. of 15-percent potassium iodide and wait 10 min. During this time interval, prepare a receiving tube; place 3 ml. of silver diethyldithiocarbamate pyridine into a 15 ml. centrifuge tube.
4. Add 5 to 6 drops of stannous chloride and mix.
5. Add 0.5 ml. isopropyl alcohol (dispersing agent).
6. Transfer sample to generator and quickly assemble.
7. Let the generator run for 45 min.
8. Read the samples at 540 mμ in the spectrophotometer. A reagent blank should be run with each group of unknowns.

Calibration curve:

A standard curve was prepared by plotting the percent transmission readings of the standards against their arsenic concentration on semilog graph paper. The values of the unknown are determined directly in microgram per gram of material, or microgram per milliliter of blood.

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[1] Association of Official Agricultural Chemists. 1960. Official methods of analysis of the A.O.A.C. Ed. 9. pp. 305-307.

Hawk, Philip B., Oser, Bernard L., and Summerson, William H. Practical physiological chemistry, Ed. 13 McGraw-Hill. pp. 848-849. 1954.

Kaye, Sidney. Gradwohl's Clinical Laboratory methods and diagnosis, Ed. 6 (Edited by Frankel. Sam, and Reitman, Stanley) C. V. Mosby Co. pp. 388-389. 1963.

[2] Liederman, David, Bowen, J. E., and Milner, O. I. Determination of arsenic in petroleum stocks and catalysts by evolution as arsine. Analyt. Chem. 31(12): 2052-2055. 1959.

Powers, George W. Jr., Martin, R. L., Piehl, Frank J., and Griffin, J. Marcus. Arsenic in naphthas. Analyt. Chem. 31(9). 1589-1593. 1959.

Winkler, W. O. Identification and estimation of arsenic residuc in livers of rats ingesting arsenical. A.O.A.C. 45 (1): 80 1962.

Results

Standard solutions that contained 2, 4, 6, 8, and 10 μg. of arsenic trioxide per gram of material were prepared and were subjected to the standard digestion method for biological material. The modified Gutziet method and modified AgDDC method were then performed on the standard solutions.

After the modified AgDDC method was completed, a standard reference curve was prepared from the spectrophotometer readings. Duplicate standard solutions that contained 3, 5, 10, and 20 μg. of arsenic trioxide per gram of material were prepared and subjected to the modified AgDDC method to determine the percent recovery. The recovery rate was found to be 86 to 94 percent with the most accurate percent recovery in the middle of the spectrophotometer scale (35 to 65 percent transmission).

The color changes of the standard solutions were read at hourly intervals for 8 hours and again at 24 hours. The color was found to be stable for 8 hours, but no longer. The lower sensitivity limit of this procedure was found to be 1 μg./g. (1 p.p.m.). Levels lower than this could be detected; however, results were not completely reproducible.

Discussion

The main advantage of the modified AgDDC was the accurate detection of levels of arsenic as low as 1 μg./g. and reproducible quantitation of arsenic in the tissues. This could be very useful when determining day to day changes in the blood of project animals and differentiating between the treatment level and toxic level of arsenic in the liver of swine that involves a difference of a microgram.

There is very little interference from other elements that might be in an unknown when the test is being run. The procedure involves equipment that could be found in any laboratory and little time is taken to set up and perform the test. Because of the stable color development, a large number of samples may be done and at the end of the working day read on the spectrophotometer. This enables the technician to perform other duties in the meantime. The procedure can be performed quickly and satisfactorily by personnel with a minimum of technical training.

Only one disadvantage was found and that was the use of pyridine. Pyridine has an unpleasant odor and after prolonged use may cause skin irritation. This was controlled by running the procedure in a hood.

The modified Gutziet method depends solely on a technician's interpretation of a yellow-brown color on a paper strip. The amount of arsenic present is demonstrated by the height of the yellow-brown color appearing on the paper strip. This method took approximately one-half hour longer to complete than the AgDDC method and had to be read immediately as the color was not stable upon standing. Fewer tests could be performed by a technician because of the time required to set up and complete the test.

The exact quantitation of arsenic and the lower levels such as 1 μg./g. could not be detected as in the AgDDC method. The standards were not stable; therefore, each day a new group had to be run.

Summary

A modified Gutziet method was compared to a modified AgDDC method in this laboratory. After reviewing the advantages and disadvantages of each method, we decided that the AgDDC test should be routinely used for determining arsenic levels in biological material, because of its superiority over the Gutziet test.

Modell, Harold, Nelson, H. A., and Ringham, Carole. Determination of nitrate in blood.

Introduction

The quantitative determination of nitrates in biological specimens has, for some time, presented problems in this laboratory. At present, a semiquantitative spot plate method utilizing a color reaction with diphenylamine is used. This method has proved less than satisfactory because it is not quantitative in all ranges of interest, and time of the technician involved for any given sample is far too great. To find a more suitable method of nitrate determination, three procedures described in the literature were considered.[1,2,3] Of these methods, the procedures of Litchfield[3] and Follett and

[1] Follett, M. J., and Ratcliff, P. W. Determination of nitrite and nitrate in meat products. Jour. Sci. Food and Agr. 14: 138. 1963.

[2] Greweling, T., Davison, K. L., and Morris, C. J. Determination of nitrate nitrogen in bovine blood, milk, urine, and rumen liquor. Agr. and Food Chem. 12: 130. 1964.

[3] Litchfield, M. H. The automated analysis of nitrate and nitrite in blood. Analyst. 92: 132. 1967.

Ratcliff[1] were found unsatisfactory. It was not possible to obtain efficient dialysis and reduction of the nitrate to nitrite in the adaption of Litchfield's method to a laboratory bench procedure; and, although not tried in the laboratory, the procedure of Follett and Ratcliff appeared to be too dependent upon pH, and it, too, involved reduction of nitrate to nitrite, a step that was to be avoided if possible. The remaining procedure, that of Greweling and others[2] which directly determines nitrates in blood was the procedure of choice.

Summary of the Procedure

Reagents:

For "Clearing" the blood

1. Sodium tungstate solution—10 percent
   Dissolve 100 g. of $NaWo_4 \cdot 2H_2O$ in 1 l. of distilled water.
2. Sulfuric acid—0.66N
   Dilute 19 ml. of concentrated reagent grade H_2SO_4 to 1 l. with distilled water.
3. Coper-silver reagent
   Dissolve 20 g. of $CuSO_4 \cdot H_2O$ and 1 g. of Ag_2SO_4 in 100 ml. of distilled water.

For Nitrate Determination:

1. Xylenol Solution
   Dissolve 2 g. of 3,4 Xylenol (3,4-dimethylphenol Eastman #1155) in 100 ml. of reagent grade acetone.
2. Sulfuric acid diluted
   Cautiously add 300 ml. of concentrated reagent grade H_2SO_4 to 100 ml. of distilled water. Mix and allow to cool before using.
3. Sodium hydroxide—0.4 percent solution
   Dissolve 4 gm. of reagent grade NaOH in 1 liter of distilled water.

Procedure:

"Clearing" the Blood

1. Transfer 2.5 ml. of blood (containing either heparin or EDTA as the anticoagulant) to a 125 ml. flask or a 50 ml. centrifuge tube.
2. Add 17.5 ml. of water and mix.
3. Add 2.5 ml. of the sodium tungstate solution, mix, add 2.5 ml. of the 0.66 N sulfuric acid solution, immediately mix, and allow to stand for 20 minutes.
4. Centrifuge until a clear supernatant is obtained and transfer 12.5 ml. of the clear solution into a 15 ml. centrifuge tube.
5. Add 0.5 ml. of the copper-silver reagent, mix, and let stand for 20 minutes.
6. Add about 0.15 gm. each of calcium hydroxide and magnesium carbonate, mix well, let stand for 10 minutes, and centrifuge.
7. Take a 10 ml. aliquot of the supernatant and proceed with the nitrate determination as described below.

Determination of Nitrate:

1. Transfer the 10 ml. of Step 7 above to a 125 ml. flask.
2. Add 0.5 ml. of the Xylenol reagent and mix.
3. Add 30 ml. of the diluted sulfuric acid, mix again, and let stand for 20 minutes in a 40° to 50° C. water bath.
4. Dilute with 10 ml. of distilled water, mix, and let cool.
5. Transfer to a 125 ml. separatory funnel, add 12.5 ml. of $CC1_4$, shake vigorously for about 30 seconds, and then allow the layers to separate.
6. Drain off the $CC1_4$ layer into a beaker, discard the aqueous layer, rinse the separatory funnel with distilled water, and allow it to drain dry.
7. *Pipet* 20 ml. of 0.4 percent NaOH solution into the funnel, add the $CC1_4$ extract, shake vigorously for about 30 seconds, and allow the layers to separate.
8. Discard the $CC1_4$ layer and filter the yellow sodium hydroxide extract through a slow paper. (Greweling suggests S & S No. 605; however, in this laboratory Whatman No. 42 was used.)
9. Measure the transmittance of the filtered solution in a Spectronic 20 spectrophotometer[4] at 420 mμ. vs. distilled water.

Discussion

Preliminary studies have indicated that the above method is a good candidate for the replacement of the spot-plate technique presently used. However, these studies have also shown that recovery of nitrate may be inconsistent. Table 1 shows evidence of these observations. It appears, however, that these inconsistencies are not due to the procedure, but rather to the dilution technique and general techniques of the investigators. Further determinations will be carried out adding a known amount of nitrate to one blood sample and using the sample for further dilutions.

See footnotes 1 and 2.

[4] Bausch & Lomb, Inc., Rochester, N.Y.

Table 1.–Recovery of nitrate

Experiment No.	Sample No.	Anti-coagulant	Microgram NO_3 ml.		Percent recovery
			Blood added	Blood measured	
HM I-15	11	Heparin	0	7.0	–
	12		500	480.0	94.6
	14		100	96.0	89.0
	15		80	77.0	87.5
	16		40	30.0	67.5
	17		20	20.5	67.5
HM I-15	18	EDTA	0	7.0	–
	19		500	430.0	84.6
	21		100	97.0	90.0
	22		80	74.0	84.0
	23		40	25.0	45.0
	24		20	18.0	55.0
HM I-15	I	EDTA	200	190.0	90.8
	II		100	100.0	91.5
	III		50	40.0	63.0
	IV		25	24.0	62.0
	V		10	23.0	145.0
	VI		0	8.5	–
HM I-19	0-1	EDTA	0	15.0	–
	0-2		0	11.5	–
	20-1		20	20.5	35.0
	20-2		20	20.5	35.0
	40-1		40	44.0	70.0
	40-2		40	37.0	70.0
	60-1		60	55.0	50.0
	60-2		60	32.0	50.0
	80-1		80	68.5	74.0
	80-2		80	77.0	74.0

The standard curve for this procedure seems to exhibit a rather peculiar inflection indicating that perhaps a chemical complexing is taking place in the 25 to 40 μg. per ml. range (fig. 1). It might be beneficial to run another series of standard solutions through the procedure to once again verify this standard curve.

Preliminary studies are presently in progress to determine if this procedure may be adapted to nitrate determination in tissues.

Summary

Methods for determining nitrates in biological materials were investigated. The literature was searched, and three possible replacements for the present method were examined. Of the three, one appeared to be a suitable procedure for the quantitative determination of nitrate in blood.

Cassidy, D. R. Studies on the stability of Unopette Disposable diluting pipettes used for counting white blood cells.

Introduction

During 1967 the suppliers[1] of the Unopette disposable diluting pipettes stopped printing the expiration date on containers of Unopettes, including those used for diluting blood before making total white blood cell counts. Hog cholera diagnosticians utilize substantial numbers of Unopettes in their work with hog cholera. We, therefore, considered it pertinent to determine the effect of aging on white blood cell counts made with outdated Unopettes beyond the expiration date. If leukocyte counts were adversely affected, the diagnostician would have no way of knowing when to discard them. To ascertain the effect of aging beyond the expiration date, the following evaluation was performed.

Materials and Methods

The study consisted of 135 total white blood cell counts. The counts were performed by three technicians on blood sampled from 15 swines. Thoma pipettes, Unopettes stored for 1 year beyond their expiration date, and Unopettes with current date were used. The data were analyzed by the analysis of variance procedure with subsequent orthogonal comparisons to extract meaningful sets. All F values were calculated, using the error mean square as the denominator with all effects fixed. All computations were performed on a computer.

Results

The results indicated that no significant difference resulted from storage of Unopettes at 4° C. for 1 year beyond their expiration date. A brief resume of the analysis results are listed.

Source of Variation	*F Values*
A - technicians	0.968
B - methods	37.958

[1] Becton-Dickinson Co., Rutherford, N.J., U.S.A.

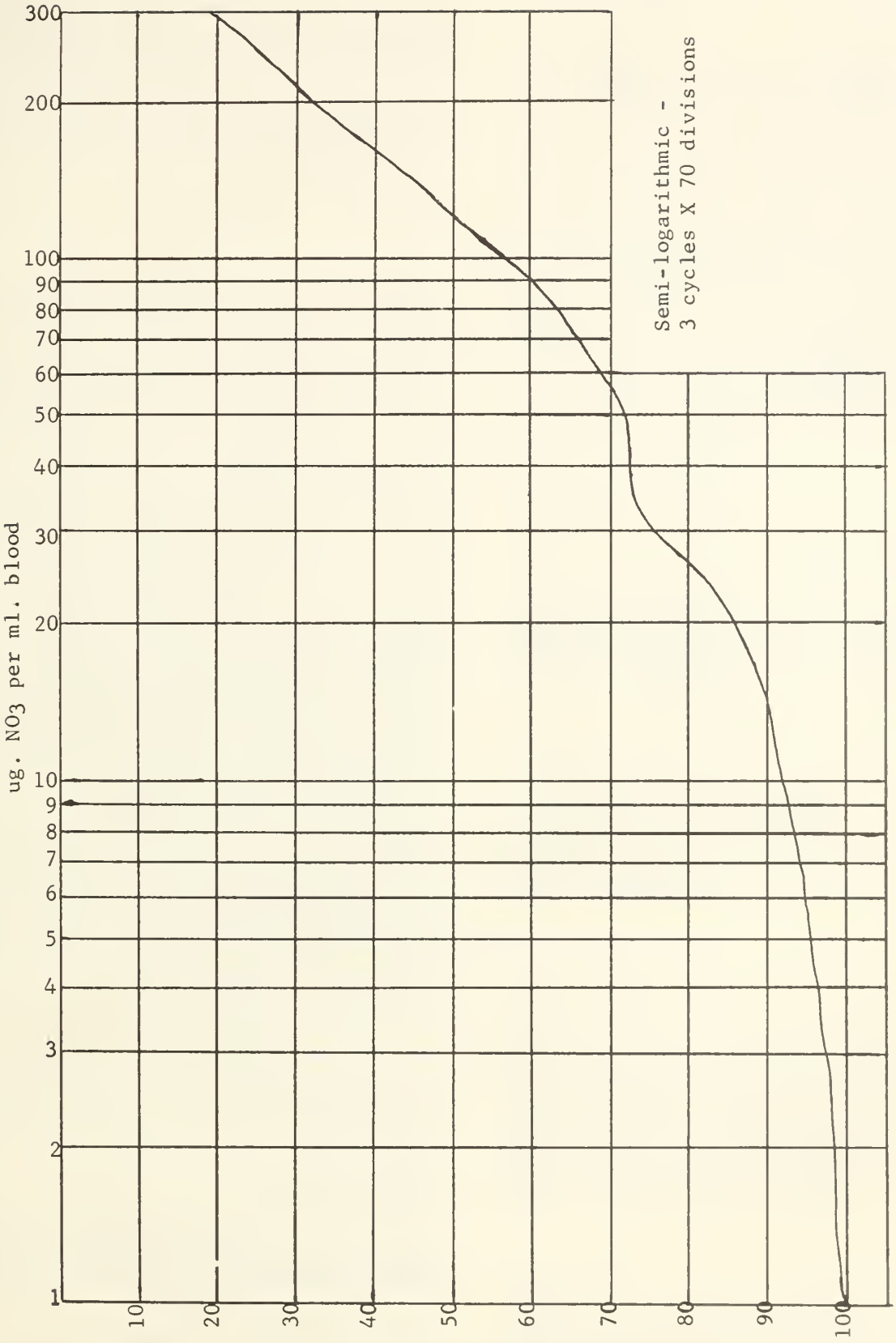

Figure 1.–Standard curve of nitrate concentration.

Source of Variation	*F Values*
AB - interaction	20.269
C - blocking for animals	5.276

Orthogonal Comparisons

B_1 vs B_2 and B_3 (C_1)
B_2 vs B_3
n. j. = 45 (Bj means based upon 45 observations)

Sum of Squares

B = 264,660,783.99
C_1 = 258,525,375.38
C_2 = 6,136,058.89
B = $C_1 + C_2$

Tests of Significance (F-tests using error mean square)

$$C_1 = \frac{258,525,375.38}{3,486,182.00} = 74.16^{**}$$

$$C_2 = \frac{6,136,068.89}{3,486,182.00} = 1.76 \text{ n.s.}$$ (Difference between current and outdated Unopettes.)

Tabular F (0.05) = 3.93 F (0.01) = 6.87

The above may be interpreted as follows: If the averages of total white blood cell counts made by both current and outdated Unopettes were identical, the probability of obtaining this much difference; for example, 6.78 by chance alone would be less than one in one-hundred. Our value of 1.76 is substantially less than this.

Discussion

These findings suggest that the diluting fluid in the Unopettes (No. 2706) evaluated is stable for up to and possibly for more than 1 year after the expiration date. It should be kept in mind that these Unopettes were kept at 4° C. during storage; had they been subjected to extremes of temperature the results may have been different.

Summary

This study indicates that the use of Unopettes beyond 1 year of their expiration date will produce no significant difference in total white blood cell counts when compared to similar counts made with fresh Unopettes.

Cassidy, D. R. and Nelson, H. A. The focal distribution of lesions of porcine hypernatremic eosinophilic meningo-encephalitis for diagnostic purposes.

Abstract

Histopathologic demonstration of eosinophils in the perivascular and submeningeal spaces of the cerebral cortex of swine ingesting toxic amounts of sodium compounds has been an extremely useful diagnostic criteria. Large numbers of swine brains for differential neuropathologic diagnosis are received by the Diagnostic Pathology and Toxicology Section of Diagnostic Services, Animal Health Division. It has been the common experience of histopathologists, examining the brains of swine that have died as the result of excessive serum sodium, to encounter focal areas of eosinophil-containing exudate in only a few slides of swine brains serially sectioned through the frontal plane. Consequently, serial sectioning of the entire cerebral cortex is frequently necessary to justify an unequivocal diagnosis. Frequently only parts or a single part of a porcine brain are available for examination. Numerous eosinophils are normally encountered randomly scattered through the brains of young pigs and are commonly observed in the inflammatory exudate in the brain resulting from infectious diseases. If specific, localized, areas of porcine cerebral cortex in which the focal accumulations of eosinophils occurred consistently could be determined, several improvements could be realized in the diagnostic procedure. The diagnosis could be accomplished quickly, with more accuracy, and less brain material would be required. The latter is frequently at a premium where sizeable amounts of brain are required for bacteriologic examination and specialized histopathologic examination procedures. A study was conducted to determine if these lesions occurred consistently in any particular area of the cerebral cortex. The results indicated lesions did not occur consistently in any particular area of the cerebral cortex.

Literature Review

The first report of swine being poisoned by sodium containing compounds was published in 1856 by Lepper.[1] Several cases reports and a considerable

[1] Lepper, H. On poisoning of pigs with brine. Veterinarian 29: 434. 1856.

number of research papers dealing with this toxicosis have been published. They are discussed by Smith.[2] The eosinophilic meningo-encephalitis in swine produced by sodium salt intoxication has been described by Smith.[3] Among the compounds capable of producing this intoxication are sodium chloride, sodium lactate, sodium propionate, and sodium carbonate. Ingestion of these compounds must be accompanied by restricted water intake to produce the typical syndrome.

Materials and Methods

Pigs were given NaCl at a rate of 2.5 g./kg. per day. The salt was administered in 8 oz. of water using a rubber tube connected to a 12-oz. dose syringe. No additional water was given. All animals were temperatured daily.

The brains were fixed by immersion in 10-percent buffered formalized saline for 96 hours and sectioned in the sagittal plane. Following paraffin impregnation and sectioning, the tissues were mounted on 50 x 75 or 38 x 75 mm. glass slides. Representative sections were stained with Harris hematoxylin and eosin.

Trial I.—Three, 30-pound pigs were given the above dosage of NaCl and H_2O for 5 consecutive days. Some water was accidentally spilled outside the pen and a small amount drained into the pen. Two of the animals were moribund and the remaining animal was ataxic on the fifth day. Body temperatures remained within normal limits. The animals were euthanized and necropsied. Gross lesions were not observed.

Trial II.—Four, 22-pound pigs were dosed at the above dosage for 4 days. Body temperatures remained within normal limits during the study although two animals became hypothermic while moribund; for example, 99.3 and 99.4. All four animals were dead on the fifth day.

Results

Focal accumulations of polymorphonuclear eosinophilic leukocytes were observed consistently in the meninges and cerebral cortices. Similar accumulations were observed, but with less regularity, in other areas of the brain. There was no constant association between brain nuclei, other brain landmarks, and the focal accumulations of eosinophils.

Discussion

The diagnosis of sodium salt poisoning in swine is complicated by several factors. During the period of excessive salt ingestion, sodium levels in the blood serum are consistently elevated, but the levels fall by the time signs of poisoning appear. The chemical demonstration of excessive sodium salt in the stomach contents is invaluable in the diagnosis; however, a negative result may be misleading since sodium is rapidly absorbed from the gastrointestinal tract. A diagnosis based on clinical symptoms is possible only if the animals are observed while showing the epileptiform convulsions, clonic contraction of neck muscles, rapid backward movement, and other symptoms associated with the toxicosis. The microscopic changes occurring in the central nervous system of swine are considered by most pathologists as pathognomonic of the disease.

Summary

The findings indicate that the focal accumulations of eosinophils in the brains of hypernatremic swine are consistently present in the cerebral cortices of swine. However, consistent, more precise localization of the lesions within these areas was not observed during this study.

Field Consultation

Sherman, K. C., Gigstad, D. C., and McDaniel, H. A. An outbreak of rabies in swine.

Introduction

An outbreak of rabies in swine occurred in Missouri during the winter of 1966-67. Rabies was initially suspected because the swine were manifesting clinical signs of encephalitis and rabies had been diagnosed in two cattle from the same farm a few months earlier. However, rabies was tentatively ruled out early in the laboratory studies since the fluorescent antibody test for rabies was negative and no Negri bodies were found on histopathologic examination of brain tissue.

Case History

The farm was located in a wooded area at south central Missouri. Forty-three almost finished swine were kept in a large woven wire pen in a timbered area.

[2] Smith, D. L. T. Diseases of swine. (Edited by Dunne, H. W.) The Iowa State University Press, Ames, Iowa. pp. 580-586. 1964.

[3] Smith, D. L. T. Poisoning by sodium salt—a cause of eosinophilic meningo-encephalitis in swine. Amer. Jour. Vet. Res. 69: 825. 1957.

The outbreak began in mid-December 1966. Approximately two pigs died each week over a 9-week period before the final diagnosis was made.

Clinical Signs

The first sign noted was slight incoordination. This was quickly followed by abnormal squealing, biting movements of the mouth, slight extension of the head, tremors, and convulsions. In every case death occurred in less than 24 hours after the first clinical signs were noted.

Laboratory Findings

Several dead animals were submitted to the Veterinary Diagnostic Laboratory, University of Missouri. No significant gross lesions were found during postmortem examination. Microscopically, marked encephalomyelitis characterized by perivascular cuffing, vasculitis, hemorrhage (fig. 1), neuronal degeneration, and neuronophagia (fig. 2). A diligent search was made for Negri bodies characteristic of rabies, but none were found. Brain tissue was also examined for rabies using the fluorescent antibody procedure. Again no evidence of rabies was found.

Mice were inoculated intracranially with the seine brain tissue to conclusively confirm or deny rabies. Rabies was not considered likely after the mice remained healthy 14 days postinoculation.

Specimens were submitted to the Pathology and Virology Sections, Diagnostic Services, National Animal Disease Laboratory, by the Missouri Veterinary Diagnostic Laboratory. No conclusive evidence of hog cholera, Aujeszky's disease, pathogenic enteroviruses, bacterial infection, or poisoning was found.

The diagnosis of rabies was confirmed following typical death of inoculated mice. The first mice died 26 days postinoculation. The fluorescent antibody test for rabies was positive on brain tissue from the mice. Negri bodies were found in mouse brain tissue.

Discussion

Meager information pertaining to rabies in swine was found in the literature. Several questions concerning this outbreak which remain unanswered are:

1. Whey were Negri bodies not found in rabid swine brains? Sections from at least six brains were examined by veterinary pathologists at the University of Missouri and the National Animal Disease Laboratory.
2. Why was the fluorescent antibody test consistently negative on the swine brain tissues? Tissues from six rabid swine brains were examined. Laboratory errors, defective reagents and equipment, errors in technique, and incompetent personnel seemed to be unsatisfactory answers since the rabies fluorescent antibody test was positive when conducted on brain tissue from mice inoculated with swine brain tissue. After the inoculated mice developed rabies, brain tissue from two or more rabid swine were examined using the fluorescent antibody procedure and still conclusive evidence of rabies was not found.
3. How was rabies introduced into the swine herd? The probable answer is a wildlife vector since the pigpen was located in a wooded area, and there was a history of rabies in cattle on the same farm several months earlier.
4. Did rabies spread within the swine herd or were all the swine bitten by rabid wild animals such as skunks or foxes?

Additional research in swine rabies is urgently needed. Personnel working with swine manifesting signs of encephalitis should always be aware of the possibility of rabies and know how to protect themselves. Persons associated with the hog cholera eradication program are especially vulnerable since many swine with hog cholera manifest signs of encephalitis.

Reference Assistance to El Salvador

E. M. Ellis made two reference assistance visits to El Salvador in 1967. The first was sponsored by AID and ANH and the second at the request of and supported by the El Salvador cattlemen. The following is a report of these visits.

BACKGROUND INFORMATION

El Salvador has a total land area of 20,000 square kilometers and approximately 2,600,000 inhabitants with a population density of 130 persons per square kilometer. It is the most densely populated country in continental America. Because of this, disease conditions in livestock used for milk and food cannot be endured. The Nation is now deficient in food with a per capita beef consumption of 13 pounds. Thirty-five percent of the national income is agriculture. Over 10 million

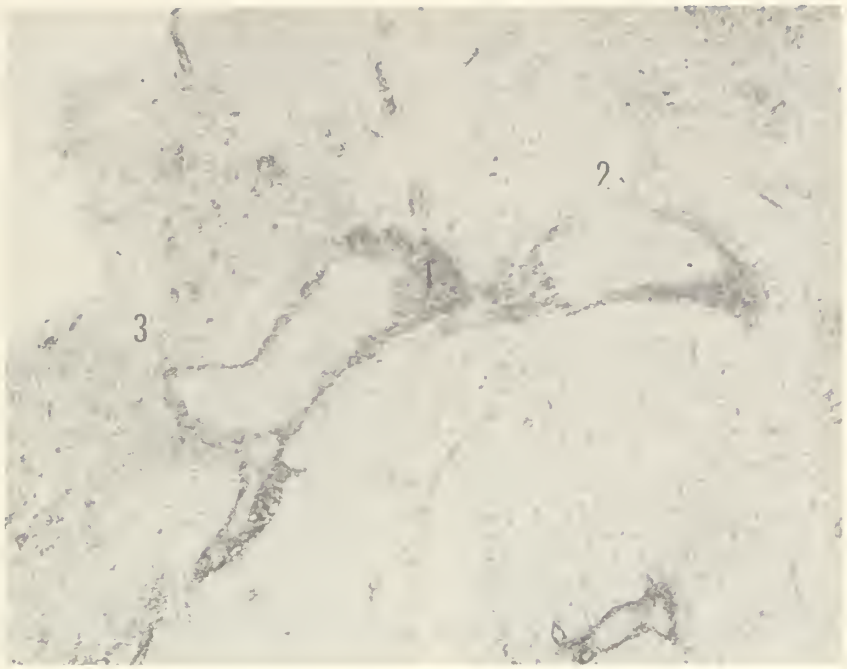

Figure 1.–Swine rabies (magnification 35X). Perivascular cuffing (1), hemorrhage (2), and diffuse infiltration with mononuclear cells (3).

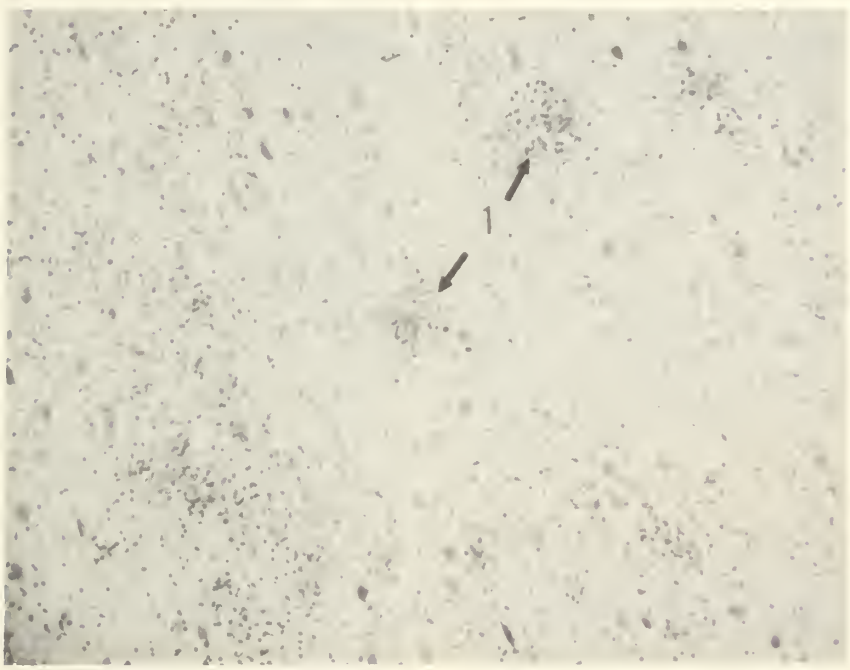

Figure 2.–Swine Rabies (magnification 100X). "Babe's Nocules" (1): Degenerating neurons are surrounded by mononuclear cells.

pounds of powdered milk are imported every year. More startling is the fact that the per capita caloric intake in El Salvador is 1,900 calories.

The deficiency in livestock production does not result from a lack of cattle but from the very low productivity of existing herds.

The national average milk production per cow is 1,500 pounds per year. Milk consumption in the city of San Salvador is estimated at an average per capita intake of 1 per 10 liter daily. People in rural areas drink practically no milk.

ASSIGNMENT IN EL SALVADOR

Atrophic rhinitis

This assignment was made in response to a request by the government of El Salvador for technical assistance in the establishment of a diagnosis for a suspected disease of swine. Some veterinarians in El Salvador believed that atrophic rhinitis existed in their swine while others believed this was not so. This disease is widely disseminated in the United States and has been a major problem to swine breeders and producers.

Rhinitis found at necropsy

The first pig examined came from Olocuilta, San Salvador, and was a native hog. The animal had atrophic rhinitis and in addition excess pericardial fluid, a roughened mitral valve, and a streptococcus was isolated. This animal had not been in association with any imported swine.

A small pig, Duroc, female of 4 months, was also infected. This pig came from stock imported into El Salvador from the United States.

Other evidence of infection was observed in herds examined. No infection was seen at the National School of Agriculture.

Cancer

A trip was made to Ahuachapan at the request of Ernesto Sahli, Director of the Mepo program, to investigate a condition in swine throught to be a form of cancer. It was said to be of a type known as sarcoma.

The possibility of this being true seemed remote but an investigation was undertaken. Several farms were visited on which animals having gross swellings at the elbow and knee joints were present. Some had large fluctuating swellings on the hip. Upon excising these, a yellow-green pus was found in abundance. A number of pigs were requested for necropsy at the laboratory. Examination of stained histologic sections of synovial membranes and pericardial sac revealed a violent inflammatory response of bacterial origin. Beta hemolytic streptococci, type A were isolated from each pig necropsied. The indicated treatment, penicillin, was administered with good results for the remaining pigs. No indication of any neoplasm was seen.

Hog cholera

Except for one herd of swine wherein the owner refused vaccination, no cholera was evident. All swine had received modified live virus vaccine except for one premise which was well isolated and where crystal violet vaccine had been used.

General husbandry

In general, herds under the direction of the Mepo program were being managed as well as any swine in the United States. The physical facilities were excellent and the programs adequate by any standards.

It was recommended; that the premises, where a diagnosis of streptococcus infection was made, should be scrubbed with 2-percent lye, washed thoroughly with water, and sprayed with a good disinfectant such as an orthophenylphenate.

Observations

The fact that the diagnosis of atrophic rhinitis could not be made and agreed upon by veterinarians at the Ganaderia is not surprising as none had had experience with this disease. However, the conditions surrounding a diagnosis of "cancer" in swine at Ahuachapan seems incredible in view of the fact that an M. D. pathologist and a veterinarian both examined histologic sections after which they made a diagnosis of "sarcoma."

Clinical signs of other swine diseases were not observed nor did any come to light during visits to swine herds. Overall, the production of swine appeared to be off to a good start in El Salvador. It was interesting to note that a market hog for domestic use weighs from 250 to 300 pounds while the desirable weight for a similar hog in the United States is approximately 210 pounds. The fat animal is valuable for its lard and for a fried-fat delicacy much in demand by the people.

Brucellosis

Laboratory.—During a visit to the Ganaderia last year, it was observed that antigen for the plate-agglutination test was outdated as much as a year. Any results using

this antigen could be doubtful. The situation had not improved very much this year. USDA plate antigen was introduced along with changes in the technique to conform with USDA standards. An example was the use of four dilutions to replace the single 1:50 dilution routinely employed. Standard droppers were also introduced. The technique now employed conforms to USDA standards. The need for Brucella antigen from the United States is very great. It is recommended that El Salvador be furnished the antigen necessary to carry out a brucellosis eradication program.

Field.—An investigation of thy level of infection caused by brucellosis was launched. Herds were found with high rates of infection. A typical herd examined using the brucellosis card test and the standard plate test constituted 256 milk cows. The husbandry was good and complete records were available. Milk production was low as is the rule in the country. Of the herd, 26 percent was positive to both Brucella tests (card and plate). The number of abortions had been increasing yearly. One animal had aborted three times but remained in the herd. A typical herd was that of Enrique Alverez y Cia, hacienda El Jobo. This herd is made up of 256 cows, all of which remain together. A study of the herd reveled an infection rate of 22 percent. To indicate the cost of removing infected animals from the herd, one may consider the following: 58 cows valued at $320 = $18,560. These cows sold at meat prices would probably bring $4,140. Therefore, the cost of removal of all infected animals totals $14,420. This loss does not include losses from abortions, reduced milk production, and other lesser costs.

Inquiry into the management aspects of the herd revealed that in 1965 there were 15 abortions; and in 1966, 19 abortions. One cow, "Polca," (no ear tags are used) aborted in 1965 and two times in 1966. "Polca" reacted to the card test and a dilution of 1:200 and greater to the standard plate agglutination test. Amazingly, she was still in the herd. It is of interest that this herd was under the observation of a private veterinarian. Insemination was carried out by using frozen semen imported from the United States—a suggestion from the first visit. In going over the breeding records it was observed that where cows were not bred after two services, they were reactors to the brucellosis tests. The herd owner kept six bulls but eliminated all but one, at our suggestion last year. The remaining bull was used only when a cow could not be bred by using frozen semen. The bull was a source of infection and it was recommended that the animal be slaughtered.

Calfhood vaccination had been practiced. Two hundred seventy-five cows had been previously vaccinated using strain 19 vaccine. The condition and method of injection were open to question. As far as could be determined, calves had been vaccinated from 10 months of age up to 4 years of age. Twenty-nine cows that had been vaccinated were positive, the majority at a dilution of 1:200 or greater.

The strain 19 vaccine used in El Salvador is imported by the Ganaderia, and cattlemen solicit the vaccination service from them. At times, when an owner has calves ready for vaccination, the Government has no vaccine or the veterinarian does not appear at the farm to vaccinate until the calves are 10 months of age or older. By this time they are infected. It was calculated that 51 percent of the vaccinated animals were infected.

Recommendations to the government of El Salvador

To proceed with an eradication program, the following recommendations were made:

1. Require all cattle in the eradication zone to be identified with an ear tag.
2. Require all cattle moving out of the eradication zone to have a health certificate showing they are brucellosis-free.
3. Require that a herd be free of brucellosis to obtain credit for purchase of animals.
4. Require calfhood vaccination with strain 19 vaccine at 3 months of age.
5. Infected cattle found in the eradication zone be branded with a letter "B" on the rear flank.
6. Cattle moving to slaughter need not have a health certificate but must be ear tagged and, if a reactor, branded.

One fact is clear. The regulations governing an eradication program in El Salvador must be simple and enforceable. The suggested ones are exactly this. Any attempt to develop a complicated system will fail because of lack of ability to enforce it. These suggestions have met with the approval of the Sub-Director General de Ganaderia.

Tuberculosis

No tuberculosis eradication program is in existence at this time. Herds here and there may be tested but with little real understanding of what is occurring in the herd. A visit should be made in which the program is started on a scale that can be maintained. The brucellosis program should be further developed before beginning a tuberculosis program.

For the second visit in 1967, Dr. Ellis journeyed to Salvador at the request and expense of El Salvador livestock producers familiar with his work from two

previous official visits. On those occasions, Dr. Ellis participated in animal health programs sponsored jointly by the U.S. Department of Agriculture and the Agency for International Development. His third trip cost the U.S. Government nothing.

The extent of El Salvador's livestock problem is reflected in the unusually low production of milk and meat products. The national average milk production per cow is only 1,500 pounds per year as compared to 8,513 pounds in the United States. Furthermore, despite a cattle population of 700,000, per capita meat consumption in El Salvador is only 13 pounds.

In an effort to pinpoint and localize the problem, a testing program was instituted covering several large herds. In one herd, 67 percent of the adult milk cows reacted to the tuberculin test. Close examination of the same animals revealed that almost 10 percent also were infected with brucellosis.

Another herd, although previously subjected to eradication procedures by the owner, was still found to be infected with both diseases (brucellosis, 0.07 percent; tuberculosis, 10 percent).

Laying out a proposed brucellosis eradication program, it was recommended that the young animals be vaccinated and that the older infected animals be slaughtered for beef.

Tuberculosis, however, presented a more difficult problem since wholesale slaughter would virtually destroy El Salvador's milk producing industry. As a first step in a long range campaign,therefore, it was urged that all milk from infected herds be pasteurized to avoid infecting humans. Pasteurization is virtually unknown in El Salvador, however; and an education program is needed to drive home the dangers of drinking raw, possibly infected, milk.

With effective disease control measures, Salvadoran cattle could help to close the calorie and protein gap between what Salvadorans have and what they need. Without disease, the healty animals could produce much more milk and meat.

The Salvador cattlemen who put up their own money for the second assistance visit are representative of the Government of El Salvador's self-help efforts to improve her agriculture. The Government has built institutions of agricultural research and education and has begun programs of land settlement, crop and livestock production, and credit for low-income farmers. A university school of agriculture and a good vocational agricultural school are turning out more and more trained agriculturists.

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